

What Brussels should do for research

The new member of the European Commission with responsibility for research, Professor Antonio Ruberti, has taken a sensible step towards a rational definition of what the European Communities should be looking for.

THERE are two ways of interpreting the decision of Professor Antonio Ruberti to seek advice on the European Communities' (EC's) research programme from three 'wise men', who met as a group for the first time last Friday. The generous interpretation is that Ruberti, unlike many of his predecessors in this post in Brussels, is prepared to consult the scientific community on its needs. The other interpretation is that the EC's research programme is in such a mess that only strong-minded people from outside the commission can impart sense to it. Both views are probably correct.

The research directorate at Brussels is also now a more manageable entity. The programmes of applied research in fields such as information technology have been spun off, leaving Ruberti in charge of science proper. (Other directorates have responsibility for the EC's collective interest in climate change, for example.) The chief component of his predecessors' legacy is the fellowship programme called "Human Capital and Mobility", much criticized for being over-lavish and badly administered (see, for example, Lennart Philipson, *Nature* **360**, 102; 1992). No doubt this programme will prompt much that is good, but nobody knows whether it is good value for money or, for that matter, whether it meets Europe's most urgent needs.

The three wise men (François Gros from France, Ilya Prigogine from Belgium and Carlo Rubbia from Italy and CERN) should urge Ruberti to step back from the turmoil of his job so as to form a view of what Europe's science most needs, and how the European Commission could most effectively assist. Of necessity, with a budget only one per cent or so of what European governments themselves spend on research, it is inevitable that the commission's influence can be only marginal. Moreover, there are the strongest reasons why the commission's support for research should not conflict with that from other sources, the European Science Foundation in particular, which is best placed to make the bridges to the East that European research needs.

Quality

One crying need is to improve the quality and enlarge the quantity of research at universities in member states that now pay conspicuously too little attention to its support. That is the only way in which the participation in research of young men and women from countries such as Greece, Ireland and Portugal will be increased. The objection that this would

seem like charitable interference (not always welcome) and that the rich countries would get nothing from it would be easily countered if it were a condition of research support that new projects should usually be collaborations with members of established groups elsewhere, and that participating institutions should be flexible about the appointment of non-nationals to their faculties. The benefits would be a greater volume of research in the short run and, eventually, a greater supply of European scientists. Who could complain at that?

Scale

At the other end of the spectrum, there are transnational projects of eminent value that national governments or their grant-making agencies are unlikely to support, for financial or administrative reasons. If the EC had come into being before CERN was created, it would have been a natural sponsor. (Even now, channelling member states' subscriptions through Brussels would be a convenient guard against currency fluctuations, but would not be acceptable until Brussels had shown that it could participate successfully in the management of large projects; the commission has an inclination to keep existing institutions alive in the face of arguments to the contrary.) More recently, the need for a centre to coordinate long baseline observations by radioastronomers cried out for EC support. The commission's still more recent involvement in the Human Genome Project is a case where the right combination of imagination and self-restraint (from the temptation to be dominant) could work wonders, for science and for the commission's reputation in research.

So there is plenty for Ruberti to do, and plenty for his wise men to say. The danger is that none of them can promise that what they set their hands to now will survive the transition from Ruberti to his successor, perhaps as soon as four years from now. In basic science, the commission's reputation has been damaged over the years by the succession of one programme by another, seemingly on whim. The difficulty seems to be constitutional: one commission cannot bind its successors except to provisions of the Treaty of Rome and its amendments. The wise men could do worse than remind Ruberti what he knows already, that almost anything he decides to do will be valuable if there is some assurance that it will last. And nothing he does will improve European science if it is stopped the minute his back is turned.

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Clinton's trade troubles

President Bill Clinton needs to make up his mind on trade even if he offends some of his supporters in the process.

THE trouble with populism is that it feeds on itself. That is what President Bill Clinton will now be sensing in his efforts to map out a strategy on international trade. During his election campaign last year, he gave US voters the firm impression that the preservation of jobs in the United States was at the front of his mind; he also talked about the need to make US industry more competitive. "Fair trade", not free trade, was the motto.

None of that was inconsistent with Clinton's fine speech on trade two weeks ago, when he urged the United States to continue to make its way in the world by being more competitive than others, not protectionist; "we must compete, not retreat" was the slogan. The difficulty is that many of those who have listened to these speeches have preferred to seize upon the forms of words Clinton used during the election to urge on his new administration new forms of protection. "Are there not American jobs to be saved?", these populists from US industry are asking, knowing that they can rally a host of allies in the Congress to that cause.

The clamour is in danger of getting out of hand, partly encouraged by conflicting signals from the president himself. Only a few days before his speech on trade, he told Boeing workers that he would take up again the long dispute between the United States and the European members of the Airbus consortium, apparently resolved for a five-year period only last year. Meanwhile, anti-dumping tariffs on European steel are in abeyance only, while some unfortunate Japanese manufacturers of light transport vehicles are penalized with entry tariffs ten times what they used to be. US semiconductor manufacturers have been persuaded not to press for a public declaration that Japan has violated the iniquitous bilateral agreement in the field by failing last year to buy 20 per cent of its microchips from US manufacturers (the figure was only 17 per cent), while the International Trade Commission in Washington is up to its eyes in solemn investigations of the trade in lentils, mackerel and in Canadian meat, against which US producers also seek protection.

These are both tedious and arbitrary ways of arranging that trade is fair. Lobbying by domestic industries suffering from overseas competition is inevitably the trigger for investigation. Everybody agrees with one of the principles of the General Agreement on Tariffs and Trade (GATT) that goods should not be exported from one place to another at prices that are unfairly low, either by government subsidy or by manufacturers seeking a larger share of a market, but that leaves ample uncertainty about what prices would be fair. The determinations made by the International Trade Commission in Washington (its counterparts in Brussels often seem as self-interested) too often fail to carry conviction in the outside world.

The United States is also among the most ardent of industrialized nations in the pursuit of what GATT calls "non-tariff restraints on trade". Industrial nations, to their collective shame, all defend themselves against imports of textiles through the infamous Multi-Fibre Agreement, whose victims are both their own taxpayers and the developing countries. But the bilateral agreement with Japan on semi-conductors, with its rules for regulating the prices to be charged in third countries as well as its quota arrangements for sustaining US exports to Japan, the various versions of the "Buy America" acts that tilt the balance of purchasing by US public authorities against supplies from overseas and the restraint on international trade in corporate shares of certain kinds (airlines, for example) are all ways of keeping out of the United States goods and even capital from elsewhere.

But does not everybody practise such stratagems? Certainly the habit is deeply ingrained. The French device, some years ago, of requiring that imported video-recorders should be tested for safety at a complex of railway sidings at Poitiers probably dented the sales of Japanese manufacturers for a while. But such tricks, not easily proved to be illegal, as well as the restrictive legislation that abounds, conflict with the underlying principle that free trade ultimately benefits all parties to the transaction. So much is proved by the way in which international trade has been the fastest growing component of the world economy for the past three decades.

So what should President Clinton do? A mood-maker if ever there was one, his first responsibility should be to seek to change the way his constituents think. Sure, there are problems about jobs, and perhaps a need for transitional assistance in some industries (not ruled out by GATT), but imported goods, if they are cheaper than the domestic kind, are also a boon and will, in the long run, free people to make products of greater value. The new president, with his emphasis on the improvement of public education, has obviously recognized the importance of preparing for that long run. He should more clearly explain his motives to those who elected him. There is a sense in which providing jobs in smokestack industries now is a recipe for making sure that there are only smokestack industries in decades to come.

Clinton should also say what he will do to rescue the almost negotiated new GATT agreement from desuetude by default. Heads of government have been meeting around the world for the past two years to proclaim the importance of this treaty, and to promise that they will exert every sinew to bring it into effect. The ambiguities that can be read into Clinton's pronouncements on trade are a licence for people elsewhere to find new causes for grumbling at what is proposed. Clinton, if he wished, could break the logjam simply by declaring that he intends to do just that. And the United States, with its enviable flexible economy, would be the chief beneficiary. Job-creation would leap ahead. So it would elsewhere, which is why the grumbles, domestic and otherwise, should be overridden.

Entrepreneur takes charge of sinking Russian institute

Moscow. The Shirshov Institute of Oceanology in Moscow has appointed a successful entrepreneur as director in the hope that he can keep the renowned institute afloat in some very rough seas.

Leonid Savostin, who owns a private geological and geophysical company with annual sales of tens of millions of dollars, has recently taken over an institute that, as with nearly all Russian scientific institutions, has fallen on hard times financially. Added to its burden is the deteriorating condition of its research fleet — including the 123-metre *Akademik Feldysh* and two deep-sea submersibles, *Mir I* and *Mir II* — and the chance that the institute may soon run out of money to operate them. For some time now the head of the submersibles programme, Anatoly Sagalevitch, has been free to strike deals with whatever organization is willing to hire the crew and pay for the fuel, but the need for ongoing maintenance and major repairs may soon force the vessels into dry dock.

The institute, run by the Russian Academy of Sciences, is no stranger to commercialism. In fact, its previous director, Vyacheslav Yastrebov, resigned last autumn after an overwhelming vote of censure. He and his colleagues were accused of mismanaging millions of rubles from exploration missions carried out by the institute's fleet that resulted in promises of payment rather than profits. A joint Soviet-French venture was said to have been put in charge of some of the institute's facilities and resources through a lease that made it almost impossible for the institute ever to regain control.

The replacement of Yastrebov, a robotics engineer, by Savostin, a geologist, surprised many at the institute. "We regard his appointment as our victory", says one researcher, "but the choice that we've made is so unusual that we still harbour some doubts."

Five years ago, Savostin left the institute to found his own Laboratory of Regional Geodynamics (LARGE). He received a contract from the institute for exploration and used it as collateral to buy a fishing boat and convert it into a research vessel. With it he competed successfully for Western contracts involving underwater search and exploration missions. He was also granted several concessions to search for gold in the waters off South Africa.

Savostin says that he decided to apply for the post of director because "I realized that the institute needed a man with my experience and opportunities". Although some were concerned that he would siphon off

likely that the money will flow in the opposite direction. The institute has already received large dividends from depositing some of its money with the company over the Christmas holidays.

Savostin says that he has not been in the job long enough to have formulated detailed plans on ways to rescue the institute, which some fear may not be able to stay open beyond the end of the year. One possibility that he has broached is an international research programme that would make use of the institute's chief asset — its fleet. The institute's good reputation in the West, he says, "is capital that is somehow underestimated in Russia", he says, and it is the duty of the director to make that asset pay off.

Vladimir Pokrovsky

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The Feldysh provides an impressive — but costly — platform for research.

institute funds for his own ventures, and even proposed unsuccessfully that the academy change its rules to prohibit any representative of a private company from serving as director of an institute, it seems more

tation in the West, he says, "is capital that is somehow underestimated in Russia", he says, and it is the duty of the director to make that asset pay off.

Foundation begins competition for survival grants to Russians

Moscow. Competition has begun for the first round of survival grants to scientists in the former Soviet Union (FSU), part of a pledge of \$100 million from US financier George Soros to support science in the region (see *Nature* 360, 617; 1992).

The International Science Foundation, based in New York, plans this spring to give \$500 to between 10,000 and 20,000 scientists who have published at least three papers during the past five years in recognized journals. The most deserving scientists will also receive as much as \$1,000 to be divided among their research team. In addition, the foundation is making available \$1 million for FSU scientists to attend scientific conferences abroad. Applicants must submit material by 31 March to be eligible for the first round of awards. The second and final deadline is 31 May.

As many as 30,000 scientists, who must be active in an area of basic science, are expected to apply for the grants. The applications will be divided into three categories according to the impact of the work as measured by citation analysis. The top group will receive individual and group grants to keep research teams intact and the middle group only individual grants; the bottom group will be reexamined using other criteria, and some percentage will be funded.

Institutions with the greatest concentration of grant recipients will also be eligible for grants to support telecommunications and other infrastructure needs. The foundation is also developing a programme to help FSU libraries that are no longer receiving important scientific journals.

The selection format, designed to provide as much support as possible as quickly as possible to the greatest number of scientists, is not without controversy. Representatives from the Baltic states lobbied unsuccessfully for a quota system to provide for geographical distribution, if not for a separate foundation. Members of the US advisory board disagreed with their Russian colleagues about the criteria for the awards, with US scientists arguing for applicants to submit proposals that would be judged on the basis of scientific merit and Russian scientists emphasizing the need to disseminate funds as quickly as possible.

Foundation officials admit that their efforts are "a great experiment" and they acknowledge criticism that such an injection of funds could undermine the existing system of supporting research. But they are confident that the money will be well-spent and that the experiment, despite facing formidable logistical problems, "is bound to be a success".

Vladimir Pokrovsky

Anticipating victory, French conservatives outline plans

Paris. The expected victory later this month for the conservative coalition in French elections would mean changes in the way in which research is funded and greater pressure on researchers to demonstrate the scientific value of their work. But the opposition party would preserve lifetime tenure and other features of a system inherited from the ruling Socialist party, and it has no clear plans to address the acute lack of mobility or the runaway spending on space research, the biggest problems now facing French science.

The fear among many French scientists is that a victory by the conservative opposition might undo the gains under François Mitterrand, who came to power in 1981. By increasing public spending, adding jobs and encouraging industry to invest in research, the Socialists made science a national priority (see *Nature* 346, 121; 1990). The policies have been sustained despite two years of harsh spending cuts under Jacques Chirac's neo-Gaullist government and the present recession; research grew from 1.97 per cent of the gross national product (GNP) in 1981 to 2.4 per cent in 1990, putting France behind only Germany, Japan and the United States.

Alain Pompidou, science spokesman for the centre-right Union pour la Démocratie Française and the neo-Gaullist Rassemblement pour la République team, says there would be no major upheavals in research. "The imperative is not to waste time on reorganization," he says, "but to improve the existing system." Pompidou, trained as a physician, is a professor of histology at the University René Descartes in Paris, a deputy to the Minister of National Education and a member of the European Parliament. He is a likely candidate for the post of science minister in a new government.

The conservatives want to concentrate scarce resources for equipment and supplies by asking researchers to bid on the basis of detailed proposals rather than giving the money to laboratories to distribute as they please. But although some public scientists would receive less, Pompidou says, civil-servant status and minimum research funding would be maintained. (Salaries account for more than three-quarters of the budget of the Centre Nationale de la Recherche Scientifique [CNRS].) At the same time, Pompidou says that not enough scientists take advantage of this security; he would like to increase competition by rewarding 'risk takers' with promotion and extra funding.

Accountability would be enhanced under a system in which projects would be evaluated "before, during and after the con-

tract is signed", says Pompidou. He believes that the present four-year evaluation of laboratories is inadequate and that expensive errors are too often detected retrospectively. But there is scepticism that such a system would work, with observers saying that research organizations would have to reshape themselves into *de facto* research councils. In addition, the opposition's proposals to enhance productivity are unlikely to cause a widespread change if employees are guaranteed tenure for life and a modest sum for research and are not required to teach.

The conservatives' recipe for internationalization rests on a large increase in the number of postdoctoral positions for foreigners and for French researchers willing to work abroad. Pompidou also wants to improve the prospects for promotion and autonomy; he says that rigidity, not low salaries, is the greatest deterrent to bright students considering a career in science.

But the opposition's plans for more postdoctoral posts would not erase the Socialist slogan, "No more French postdocs in France". In a country proud of its policy of lifetime employment, the slogan reflects a reluctance to create a population of researchers on short-term contracts. But this reluctance has impeded the mobility of young researchers within France and the flow of talent into active research groups.

Similarly, the opposition's manifesto contains few concrete proposals on industrial research, aid to universities or disproportionate spending on space. It has presented no new plans for wealth creation, apart from vague incentives to stimulate the mobility of researchers between public research and industry. The Socialist minister for research and space, Hubert Curien, has encouraged industry to do more research through subsidies and tax credits, but after a rise in research financed by industry from 0.94 per cent of GNP in 1984 to 1.1 per cent in 1990 the growth has levelled off and fallen short of the government's target of 1.5 per cent.

Although the opposition wants to transform the centralized university system by creating autonomous research-based universities, it excludes merging the ministries of national education and research and space. But universities are unlikely to receive more research funding without such a merger. Moreover, the opposition's proposals contain none of the strong-arm measures needed to make full-time researchers assume some teaching duties.

Pompidou claims that the Socialists have used secret military expenditures to overestimate increases in civil spending, the same accusation used by the Socialists in 1988



Declan Butler

Pompidou sizes up science.

against the outgoing conservative government. But the opposition has taken no public position on the rapid growth in spending on space research, which has risen from 6.4 per cent of the civil research budget in 1982 to 18 per cent this year (see *Nature* 359, 659; 1992). Pompidou says that firm decisions on space spending and all other areas would come after a survey of the entire research system.

Declan Butler

US plans search for ITER site

Washington. US Department of Energy (DOE) officials expect by the end of the summer to have a plan for choosing a US candidate site for the proposed International Thermonuclear Experimental Reactor (ITER) (see *Nature* 358, 269; 1992). Each participant — the United States, Japan, Russia and the European Communities — has been asked to submit its choice by December 1994, with a site to be chosen by the following December.

DOE officials have yet to decide on a selection process, for example whether to limit the field to DOE's national laboratories or to hold an open competition as was done for the Superconducting Super Collider. The disadvantage of the latter approach, says Thomas James of the department's Office of Fusion Energy, is the time and expense of preparing a proposal that, even if selected by US officials, has only a one-in-four chance of winning. The site must also meet all US environmental and legal requirements.

At present, the ITER partners are assembling teams at the project's three joint centres in Garching (Germany), Naka (Japan) and San Diego, California. A decision on whether to proceed with building the test reactor, planned for operation as early as 2005, is not expected until at least 1996.

Tony Reichhardt

International manufacturing effort begins with six projects

Tokyo & London. The first real test of the Intelligent Manufacturing Systems (IMS) project, an international effort initiated by Japan to develop the automated factories of the future, got under way last month following years of negotiations between government and industry officials in Japan, the United States, Europe, Canada and Australia. Some 140 industrial partners in companies, universities and research institutes will participate over the next year in six pilot projects to test the feasibility of international collaboration in the research and development of new manufacturing systems.

In 1989, Professor Hiroyuki Yoshikawa, then dean of the faculty of engineering of Tokyo University, won the backing of the Ministry of International Trade and Industry (MITI) for an international research centre in Europe or the United States. But government officials in Washington and Brussels reacted coolly because of the proposed central administration by Tokyo and because the Japanese solicited research proposals before an international agreement on the project had been reached. Under US and European pressure, Japan agreed to return the proposals to their country of origin pending completion of a framework for the project.

The negotiations have taken three years, during which time the project has been joined by Canada, Australia and five members of the European Free Trade Association (Austria, Finland, Switzerland, Sweden and Norway), as well as by the United States and the European Communities. And there have been

modifications to the original plan.

Instead of one central research institute funded largely through Tokyo, the IMS test projects are decentralized, with each of the six participating regions organizing their own research consortia (see table) and funding mechanisms. The structure is similar to the European Strategic Programme for Research in Information Technologies (ESPRIT).

One sign of Tokyo's lessening control over IMS is that only four of the six test cases have Japanese representation. The first — "clean manufacturing in the process industry" — covers continuous manufacturing processes in the chemical industry rather than the batch assembly processes that Japanese industry had expected IMS to examine, and the Japanese partner, Toyo Engineering, was brought into the consortium relatively late.

A report released last month by the British Institution of Electrical Engineers estimates that about £10 million (US\$14.5 million) will be spent by all regions on the test cases over the coming year. Keichi Kawakami, deputy director of MITI's Industrial Machinery Division, expects that Japan will spend about ¥600 million (US\$5 million) on the four test cases that Japanese companies are participating in, half from MITI and the rest from industry. The US government is not contributing any money to the pilot projects, although officials say that several government agencies have "been watching IMS closely" and would welcome requests for support from

Japans funds domestic IMS

Tokyo. Apart from international aspects of the Intelligent Manufacturing Systems (IMS) project (see left), a substantial domestic effort is already under way in Japan.

Since 1990, about 65 leading Japanese companies from the electronics, shipbuilding, car manufacture, steel, construction and robot-manufacturing industries have spent nearly ¥800 million (US\$7 million) a year to fund a domestic IMS feasibility study. These funds are matched with a similar contribution from MITI that is separate from the approximately ¥300 million that the ministry will invest in the international test cases.

The core companies and academic research organizations initiated 21 feasibility study projects in 1991-92. These projects and the mix of participants were radically revised for a second phase of 24 projects in 1992-93. **D.S.**

industrial consortia. European companies will receive some support from the telecommunications and information technology directorate of the European Communities, of which ESPRIT is a part.

The programme will be monitored by a steering committee, technical committee and intellectual property rights committee, which met last week in Sydney in the first of a series of meetings to be held this year. A decision will be made early in 1994 on whether the full IMS programme should be launched and, if so, what form it should take.

David Swinbanks & David Dickson

IMS test cases and lead partners in each region

	EC	EFTA*	USA	Canada	Japan	Australia
Clean manufacturing in the process industry	ICI Engineering	Finnish Forest Industry Federation	DuPont	Abitibi-Price	Toyo Engineering	
Global concurrent engineering	Technology Transfer Plc		North Carolina State University	Northern Telecom		
Enterprise integration for global manufacturing	British Aerospace Defence	Ahlstrom Machinery	Newport News Shipbuilding	Toronto University	Toyo Engineering	CSIRO*
Holonic control systems	Softing GmbH	Softing GmbH	Allen Bradley	Queens University	Hitachi Ltd	
Rapid product development	Daimler-Benz AG		United Technologies Corp.	Pratt & Whitney Canada		
Knowledge systematization	ADEPA*	Asea Brown Boveri	Deneb Inc.	Alberta Research Council	Mitsubishi Electric	

* EFTA: European Free Trade Association; CSIRO: Commonwealth Scientific and Industrial Research.

Source: IMS programme

Events are bringing change to US Antarctic research

Washington. The US Antarctic Program, striving for balance between scientific exploration and environmental protection, may be headed down a new path that would greatly affect research on the continent.

In anticipation of a higher profile for the \$240-million a year programme, the National Science Foundation (NSF) has elevated its status from a division within the geosciences directorate to a programme within the office of the NSF director. Cornelius Sullivan, an oceanographer at the University of Southern California, has just been named to head the office, succeeding Peter Wilkness, who is stepping down after eight years (see below). At the same time, the US National Academy of Sciences is preparing a report for the State Department analysing the impact on science of a new international agreement on the environment, the end of the Cold War and growing interest in global climate.

Not surprisingly, all of these changes have made scientists apprehensive about their status. "Antarctica has always been considered a great big playground for scientists", says Susan Solomon, a member of the academy committee and a government scientist who studies ozone depletion over Antarctica. "But we've learned that we'd better not throw any more candy wrappers on the ground."

The immediate concern is the pending interpretation of an international environ-

mental protocol adopted in October 1991 by the 26 countries with significant Antarctic research programmes. Although the US Senate approved the protocol last autumn, the State Department has decided that legislation is needed to spell out how its provisions

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Human and animal life share tight quarters.

should be enforced.

A major question to be decided is the choice of an agency to regulate and monitor activity on the continent. The Bush administration last year supported a bill that would leave NSF in charge, but environmental groups favour giving regulatory authority to such agencies as the National Oceanic and Atmospheric Administration and the Envi-

ronmental Protection Agency.

Testifying on 23 February before the science subcommittee in the House of Representatives, NSF officials argued that the agency has the necessary expertise to do both science and environmental protection and that it is more efficient to let one agency coordinate both activities. But environmental groups say that NSF has had a sorry record of stewardship over the past 35 years and that there is an inherent conflict in an agency carrying out research and then certifying that its actions are legal.

Antarctic researchers are especially fearful of administrative obstacles that may delay or cancel experiments, including changes that might prevent them from responding quickly to scientific opportunities or regulations that could prohibit current activities.

A bill (HR 964) that takes NSF's side on most issues is sponsored by Representative Rick Boucher (Democrat, Virginia), chairman of the subcommittee that conducted the hearing. The Clinton administration has yet to take a position, although an interagency task force is expected to reach a decision by the summer.

At the same time as NSF is fighting to retain control of US activities in Antarctica, it is negotiating with the US Navy over the military's desire to reduce or eliminate its historic role of providing logistical support in Antarctica. The end of the Cold War and the decline in the US military budget is forcing the Defense Department to reexamine its presence, say Navy officials, and its 780 personnel may be needed elsewhere. The Navy has already convinced NSF to take over disposal of hazardous waste; starting on 1 October, a civilian company will perform those duties. In another change, US research institutions will be forced to dispose of low-level radioactive wastes generated by their scientists while working in Antarctica.

The US State Department is also interested in taking a fresh look at the Antarctic programme. The department has asked the National Academy of Sciences to report in June on the impact on science of the changing geopolitical climate and the increasing global concern about environmental issues.

National sovereignty has long been an important consideration in Antarctic exploration and State Department officials believe that research agendas will continue to be shaped by the desire of individual nations to maintain a presence. At the same time, Antarctica may hold the answers to important climatic questions.

"Tensions over the relationship between science and government policies are not new", Tucker Sculley, a State Department official, told the academy committee at a meeting last month. "But we have new players and new circumstances. And that puts a greater burden on scientists to be clear about their contribution."

Jeffrey Mervis

Spreading the word

As an oceanographer with 13 expeditions to Antarctica and five to the Arctic, Cornelius (Neil) Sullivan thinks that he understands what it means to do research at both poles. But applying that knowledge to fund the best science is only half of his new job as



Neil Sullivan

head of the Office of Polar Programs for the US National Science Foundation (NSF).

"We [scientists] need to present a much better picture to the US public of how polar science affects their lives", says Sullivan, who directs the Hancock Institute for Marine Studies at the University of Southern California. "The New Zealanders, for example, have a fine appreciation of Antarctica and what it means to them, and we need to send a similar message to students, to our colleagues and to Congress."

Sullivan says that increased concern about such environmental issues as global warming and the ozone hole has created a receptive audience for his message. But greater visibility is a mixed blessing: "Being in the director's office means that any successes are noticed", he says. "But of course, there's an obvious downside to that."

At the age of 49, Sullivan is prepared to work for half a dozen years or more to make an impact on the \$240-million programme. "It'll be two years before you can even see my fingerprints on the budget", he says, "and in any large organization, it takes quite a while to really do something. But I know I'll never be bored."

J.M.

Britain forms panel to study barriers to women scientists

London. The British government has asked a group of women scientists to study what can be done to lower the barriers faced by women in science and technology.

The working party will be chaired by Nancy Lane, lecturer in cell biology at the University of Cambridge, who has already been advising the prime minister, John Major, on the government's proposed Citizen's Charter. It will make its proposals to a new Cabinet Office Committee on Women in Science and Technology chaired by William Stewart, the government's chief scientific adviser.

In announcing the government's decision to set up the new committee, William Waldegrave, the cabinet minister responsible for science and technology, acknowledged last week that women scientists face more daunting career prospects than their male counterparts — if they do not drop out altogether. "It is obvious that we are not using the resources of half of our people properly", Waldegrave said.

So far, however, the government appears reluctant to push for aggressive measures to

change this situation. Waldegrave said he opposes quotas for fear of "ghettoizing" women in science and prefers an approach taken by the medical profession that has encouraged more women to become doctors primarily by changing its image.

Although the situation is improving, the prospects for women remain relatively bleak. At the undergraduate level, for example, women are well represented in biology but poorly in other areas — particularly mathematics, physics and engineering — where they make up only one-third of undergraduates.

In addition, the problem gets worse as their careers progress. In the Civil Service, for example, women make up 35 per cent of those at the assistant scientific officer grade but only 20 per cent of higher scientific officers and 9 per cent of senior scientific officers. There are thought to be only two female professors of physics and one of chemistry in British universities.

Arguing that equal-opportunity legislation is not enough, women members of various professional organizations have sought programmes that recognize, for ex-

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Waldegrave, Stewart and Lane

ample, the importance of adequate child-care facilities. The Agricultural and Food Research Council, for example, has adopted measures ranging from special courses designed to equip women for senior positions to an agreement to increase the numbers of women in senior grades by at least 5 per cent a year over the next five years.

Other institutions, however, have been less responsive. "One problem is that men may not entirely understand the barriers that women scientists face," says Lane.

The working party will hold a one-day public meeting at the Royal Society in London at the end of May. Its report is due to be published in the autumn, and a reference to its work is likely to be made in the forthcoming White Paper on the organization of science.

David Dickson

Woman at NIH trail men in salaries and tenure

Bethesda, Maryland. Women scientists working at the US National Institutes of Health (NIH) are granted tenure less frequently and earn less than their male colleagues, according to a new report by a task force looking at gender issues on the NIH campus. Although women make up about 30 per cent of the NIH fellows in the past decade, they represent only 18 per cent of newly tenured researchers during that time.

In addition, a preliminary analysis shows that men earn between \$1,000 and \$6,000 more in basic pay than women of the same rank.

The disparities are the result of a lack of uniform policies for tenure, promotion and pay scale at NIH, according to a task force set up in 1991 by the NIH director, Bernadine Healy, and reorganized this past summer. It is difficult for junior researchers to know how best to advance their careers because the scientific directors of NIH's 15 institutes and centres exercise considerable control over that information, which often is not available in print. Hynda Kleinman, chair of the task force and a developmental biologist at the National Institute for Dental Research, says the fact that so many senior women scientists have husbands who work at NIH is evidence not of nepotism but of the difficulty of obtaining the necessary career information through normal channels.

Only about 5 per cent of NIH fellows obtain tenured positions, but the chances for women are made worse by their low visibility and the attitudes of their male

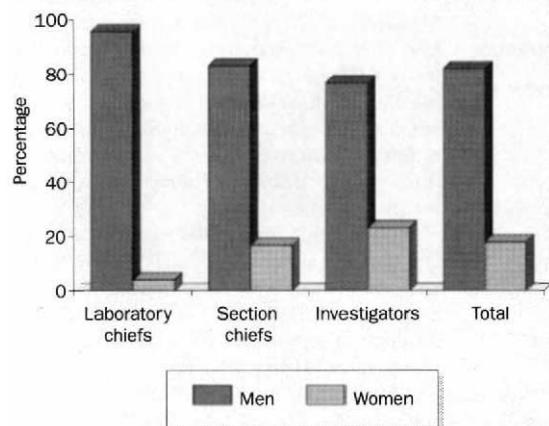
peers. Name recognition is an important factor in tenure decisions, says Kleinman, but women are invited to outside meetings less often than their talents merit because of an informal 'old boys' network'.

NIH officials have in general responded positively to the recommendations made by the task force and by other recent internal reviews. Lance Liotta, deputy NIH director for intramural research, says that comments will be sought on a draft procedure for awarding tenure to appear in the next issue of a newsletter begun recently by NIH to improve internal communications. Last year, almost half of those receiving tenure were women, an improvement that Kleinman attributes to the greater emphasis placed on the issue by Healy and to the abundance of eligible women who were denied tenure in previous years.

A council of women scientist advisers elected from each institute has been formed to discuss these issues and to improve communication between laboratory chiefs and women scientists as well as among women at NIH. One adviser has asked for job descriptions from each woman in her institute to encourage their participation on panels and at meetings. There are also plans for a new lecture series named for a woman that will feature women scientists at NIH.

Jenna Roberts

A tenuous hold on tenure at NIH



Source: NIH Office of Education

Economic realities lead to reductions at JPL

Washington. Responding to shrinking business and overcrowding, the US Jet Propulsion Laboratory (JPL) in Pasadena, California, plans to cut its work force of 7,500 by about 1,000 over the next five years. Although most of the cuts will come from attrition and slower hiring, some redundancies are also likely; in addition, the plan calls for closing down rented offices and consolidating workers on the main campus.

The size of the cuts signals a changing role for the laboratory, which has managed nearly every US solar system exploration mission for the past 30 years. Although JPL managers as well as outsiders see the move as an appropriate response to the recession in the US aerospace industry, centred on southern California, it also marks an end to an era when JPL could depend on a steady stream of billion-dollar projects such as Viking, Voyager and Galileo to keep its engineers and scientists busy. The National Aeronautics and Space Administration (NASA), which contracts JPL's services through the California Institute of Technology, has made it clear that there will be smaller, cheaper planetary missions in future, and these projects will require fewer staff.

The shift to smaller missions, no easy task in itself, will also eliminate JPL's monopoly on planetary exploration. NASA has hired the Applied Physics Laboratory of Johns Hopkins University near Baltimore, Maryland for one of its first low-budget 'Discovery' missions — the Near-Earth Asteroid Rendezvous (NEAR) — marking the first time a planetary project has been handled by a non-NASA centre. In fact, one of the goals of the Discovery programme is to involve more industry and university teams as principal contractors. NASA is under political pressure to help the beleaguered US aerospace industry, and one way of doing that is to transfer to industry contractors the work traditionally done at centres such as JPL.

With new competition for a dwindling number of planetary missions, the laboratory must either scale back or find new business. In the 1980s, the rapid buildup in the US military and the large and complex planetary missions led to more staff. But those opportunities are gone, and JPL may well begin to look as it did in the 1960s, when smaller spacecraft such as Mariner and Pioneer were being launched and its work force was smaller.

If that occurs, the reduction of 14 per cent in its work force may help JPL to survive lean times; other NASA centres, full of civil servants and protected by home-state politicians, are more resistant to pruning. By comparison, President Bill Clinton's plan to shrink the federal payroll by a quarter requires NASA to cut only 1,000 of its 25,000 federal workers by 1997 — a reduction of 4 per cent.

Tony Reichhardt

Indian budget favours space, atomic energy

New Delhi. Indian scientists say that an increase of US\$100 million in a research budget of \$1.3 billion for 1993–94 announced last week by the government falls far short of meeting the needs of science. Most of the increase is earmarked for atomic energy and space, and the budget of the University Grants Commission has been reduced. In addition, science administrators have complained about the increased competition to national laboratories from foreign technologies now allowed into the country, and the lack of money for developing technologies to strengthen Indian industry. The government has proposed an income tax exemption on contributions from industry to national laboratories to help those institutions bridge the funding gap.

K.S. Jayaraman

US purchase of British computer raises controversy

Washington. A recent decision by a US government weapons laboratory to buy a massively parallel computer from a British-owned company has touched off protests from US supercomputer manufacturers and raised questions about trade policy in the competitive international computer market. The Lawrence Livermore (California) National Laboratory, which is government-funded but privately operated, is negotiating a contract reportedly worth \$15 million with Meiko Scientific Corporation of Waltham, Massachusetts, a wholly owned subsidiary of Meiko World Ltd of Bristol, for a computer to be used in weapons-related research.

The planned purchase has generated controversy on two counts. Critics, including US companies who bid unsuccessfully, are upset that the government would buy from a non-US company — even though there is no law forbidding it. But David Schwoegler, a spokesman for Livermore, says that the machine in question was designed entirely in the United States and that 85 per cent of it was built there. Meiko has sold computer equipment to the US government in the past.

Critics also regard the 1991 sale by Meiko of advanced computer equipment to an Israeli university as a breach of a US–Japanese agreement restricting the sale of computers that might be used for weapons design. In this case, however, the sale was made by the British company, which was not subject to any such restrictions.

Jonathan Streeter of the US Department of Commerce admits that it is difficult to define a 'US company'. In the case of the sale to Israel, Meiko was acting as a British company while with Livermore it is asking to be regarded as a US company.

Danny Hillis, chief scientist for Thinking Machines Corporation of Cambridge, Massachusetts, one of the losing bidders, says that his company is upset not on the basis of trade policy but rather because Meiko is selling Livermore a computer that has not as yet been developed, as the rules require. "As near as we can tell, the machine [that the lab is buying] doesn't exist", he says.

Schwoegler denies this accusation, saying that the machine is "off-the-shelf". A spokesman for Meiko refused to say whether the computer being sold to Livermore is in production or whether there have been previous sales. Hillis said that his company would reserve judgement on whether to file a formal protest until the terms of the contract are made public.

Tony Reichhardt

London meeting on British science

On Friday 19 March, there will be a meeting at the Royal Society, 6 Carlton House Terrace, London SW1, to discuss proposals for the forthcoming White Paper on the organization of British science. The speakers will be Sir Eric Ash (Rector, Imperial College London), Professor Michael Brady (University of Oxford), Dr Dai Rees (Secretary, Medical Research Council) and Sir Mark Richmond (Chairman, Science and Engineering Research Council); the meeting will start at 9.30 a.m. and end at 4.00 p.m.

Admission to the meeting will be by ticket, free of charge, obtainable from Mary Sheehan, Nature, 4 Little Essex Street, London WC2R 3LF. Coffee and tea will be provided.

There will also be a sandwich lunch for those who want it, **at a cost of £5:** please send a cheque made out to *Nature* with your ticket application.

New Zealand risks brain drain with new funding system

Sydney. A new competitive system in New Zealand for allocating government funds to science, part of an increased emphasis on applied research, has sparked fears that the shift could start a 'brain drain' out of the country.

The system, which imposes an annual review of projects, involves a new structure for supporting science that serves the public good. It replaces one in which money was distributed through individual departments with a minimum of outside review. In addition, it dissolves the Department of Scientific and Industrial Research (DSIR) in favour of ten government-owned companies known as Crown Research Institutes (CRI).

The changes, together with significant retrenchments in certain areas, have caused great concern among government scientists in a country where private sector spending on research is very small. That situation, combined with salaries that are NZ\$12,000–NZ\$30,000 (US\$6,000–US\$15,000) higher just across the Tasman Sea in Australia, will certainly tempt many scientists to look elsewhere for better jobs.

A recent report by the Science Minister's Science and Technology Expert Panel warned of the possible loss of 328 scientists in various fields this year unless the government or the private sector increased investment in research. That would be 10 per cent of the country's estimated population of active scientists. The Ministry of Agriculture and Fisheries made 100 scientists redundant in the past year, while the recent reorganization had resulted

in 97 scientists being laid off.

The government believes that staffs will grow once the reorganization has been completed, with additional researchers hired in areas favoured by the new funding arrangements. But Alan Kirton, president of the New Zealand Association of Scientists and a meat researcher with one of the new crown institutes, says it is unlikely that those who have lost their jobs could be retrained to take up new positions.

The controversy has arisen from two separate policy changes made by the National Party (conservative) government after its election in 1990. The first change placed almost all government research funds, apart from NZ\$46 million for university research plus the money for defence and medical research, into a separate Public Good Science Fund (PGSF). DSIR scientists and those working in the Ministries of Agriculture, Fisheries and Forestry and the Meteorological service now compete for a share of the NZ\$240 million in the new fund.

Researchers submit proposals to the PGSF, which allocates funds according to both merit and a previously agreed percentage distribution of funds between various economic sectors. The total amount of money remains unchanged, but some areas have been assigned a higher priority.

The second change, which occurred last July, pooled all the scientists in the DSIR and ministries into 10 government-owned companies with a total of 3,000 science staff. The CRI researchers will still compete for funds, but now they will be freed from

the constraints of public service rules that made it difficult to receive royalties from inventions.

But the major change remains the new funding priorities, which push scientists closer to industry and primary production. The big winners are those involved in primary production — including sheep and forestry — while the losers are those engaged mainly in basic research.

Scientists have found it difficult to deal with the extensive documentation required and the economic jargon used by the PGSF. At one point the science ministry even produced a booklet on the special economic terms to help scientists.

One advantage of the new system is awards of greater length, some as long as five years. But the additional review places heavy demands on time, leading to wry comments that one of the few growth areas in New Zealand science is in science policy.

Many scientists believe that the changes go too far. John Peat, a chemical engineer at the University of Canterbury, believes that the DSIR needed to be made more efficient and relevant to industry but that the reforms have ignored the needs of scientists. It is not possible to fund all research according to preset economic goals, he says, without unduly restricting the efforts of those in the laboratory. Another unfortunate result of the reorganization, he adds, is an increased emphasis on "short-term, fast buck" projects to the detriment of longer, open-ended projects that might reap far more substantial results.

Mark Lawson

NEWS IN BRIEF

Basel. Switzerland last week voted by a 2:1 margin against a total ban on the use of animals in research. This is the second time in two years that animal activists have failed in a referendum to curtail or abolish animal experiments. Switzerland has some of the strictest regulations in the world on the use of research animals. After last year's referendum, the government acknowledged the public concern by requiring that each experiment must be approved individually. However, it was no surprise that voters failed to give their approval to a complete and immediate ban on all animal experiments.

The total number of animals used in Switzerland, home to several large pharmaceutical companies, has fallen sharply in recent years. For example, Basel-based Hoffmann La Roche now uses only 20 per cent of the number of animals it used in 1980.

O.K.

Munich. The International Council of Scientific Unions (ICSU) has asked CERN, the international particle physics laboratory in Geneva, to invite back four Serbian scientists expelled to honour UN sanctions forbidding cooperation with Serbia (see *Nature* **361**, 483; 1993). ICSU, which represents more than a hundred scientific organizations, argues that the sanctions do not apply to noncommercial science and that they "violate the principles of universality of science". All ICSU members have signed an agreement to abide by this principle.

CERN has a tradition of separating politics from science, but it is not a member of ICSU. The matter will be taken up next week at a council meeting of member states, and CERN says that its status as an intergovernmental rather than a nongovernment entity complicates the issue.

A.A.

London. This year's Australia Prize, worth \$A250,000 (US\$175,000), has been awarded to Horace Barlow of the University of Cambridge, Vernon Mountcastle of Johns Hopkins University Medical School in Baltimore, and Peter Bishop, formerly at the Australian National University and now at the University of Sydney, for their work on sensory perception. The publicly funded prize was first awarded in 1990 at the suggestion of the Labour government's former science minister, Barry Jones, who argued that an international award for outstanding scientific achievement that also promotes human welfare would help to raise the status of science both among the Australian public and in the international research community. Next year's prize will be awarded for research in sustainable land management.

D.D.

Washington. John Diggs, head of extramural research at the US National Institutes of Health (NIH) since 1990, is leaving in June to become vice president for biomedical research at the Association of American Medical Colleges (AAMC). Diggs, a physiologist who has been at NIH for 19 years, says that the biomedical research community needs to become more aggressive in claiming its share of the federal budget. "We are losing ground to the hard sciences", he says about funding trends since 1985. "We have to educate the public better in explaining how research pays off in saving money and improving the health of Americans." Diggs replaces Thomas Malone, who is retiring; Malone joined AAMC in 1982 also after leaving NIH, where he had been deputy director.

J.M.

Iceland out in the cold

SIR — Having enjoyed British post-graduate medical and scientific training and collaboration from 1968 to 1981, I read with interest your survey on Nordic Science (*Nature* 360, 511–529; 1993).

To my surprise and disappointment, Iceland, traditionally regarded as a member of the Nordic group of nations, was excluded without explanation. While understanding the necessity of being selective in such a survey, I cannot quite appreciate such dismissal of my mother country, the smallest in terms of population but one that has the world's longest democratic tradition.

Although there is room for much improvement in Iceland as regards funding and political understanding of the importance of scientific research as an essential ingredient of our livelihood and survival as an independent people, it does not justify your ignoring completely in this context significant scientific contributions by a well educated people.

My anger got dispersed by the comforts of Christmas but the issue of 21 January (*Nature* 361, 194; 1993), brought an apology that made me reach for my pen. Your survey showed two maps, a satellite view across Scandinavia on the cover, and a map of Scandinavia, Denmark and Finland together with adjacent continental European countries on page 510. On both, Iceland, usually an undoubted member of the Nordic countries, is not shown. Surprisingly though, when *Nature* does see reason for correction, it is to apologize for the omission of Poland from the map!

The days of great geographical explorations are over. Instead we are now faced with exploring the world of possibilities offered by natural resources and the ingenuity of our minds. Scientific ideas and communications are not and should not be limited by manmade boundaries. But as we are "heading into an uncertain future", preservation of and respect for cultural diversity may ensure fertility of ideas and a nation's size may be irrelevant to the importance of its contribution in this respect.

Guðrún Agnarsdóttir

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Consent laws

SIR — I refer to the article (*Nature* 361, 102; 1992) by Declan Butler concerning the work on French children born by artificial insemination by donor (AID).

He states the issues succinctly by quoting the views of Claude Paoletti, director of life sciences at the CNRS, and Jean Bernard, the former president of the (French) national bioethics advisory committee, to the effect that present laws put researchers in a dilemma. I hope that scientists in the European Communities will take cognizance of these views in framing the ethics for research on human populations.

Regrettably, I was in India when Butler visited our laboratory, so did not have the opportunity to discuss this study (of which I am the first author). The description given to Butler of some aspects needs to be clarified. Given the controversy in French newspapers relating to our research it is absolutely necessary that facts in a scientific journal be correct.

It may help researchers to understand the possibilities and limitations of our work if I state clearly its purposes. The quality of data on the cognitive traits of children is variable. Questions relating to the data of Sir Cyril Burt have not helped matters. I and my colleagues wish to provide the scientific community with reliable data on cognitive traits of related children. Given reliable data, the understanding of cognitive and genetic processes may be a little easier. Both the choice of the cognitive trait and the method are important. A trait that is not stable in the population under study is not a suitable object for psychometric or genetic analysis. The relevance of the half-sib method for complex traits is well established in quantitative genetics. It has not been used for the study of cognitive traits because of the difficulty of finding half-sib samples and, when they are found, some environmental factors are invariably involved. To avoid confounding these factors, we decided to study children born by AID.

Our study was in two stages: (1) to find which of the available tests would be appropriate for use on French children by using sibs as well as unrelated children and (2) to extend our previous study of cognitive traits on sibs to half-sibs by using the tests selected for their reliability and appropriateness for analysis.

In the current 'half-sib' study, our tested sample consisted of about 3,100 children aged between 7.5 and 14 years, of which 103 are half-sibs produced by AID and 103 were in the control group. Clearly, with these samples, there was no way we could assess "pre- and post-natal effects of artificial insemination . . . on the children of the same father". The research is not yet complete. After its completion, if the statistical analysis shows differences between the children born by AID and the control group, we shall then look into the

causes of the differences (see, with respect to adoption ref. 1).

With our design, it is not possible for us to "test whether assisted procreation itself could cause harmful neurological or psychological effects". The half-sib method gives us the opportunity to estimate the additive effect of genes causing the resemblance among the half-sibs of the same donor reared in different environments in probabilistic terms. The estimate of genetic parameters is less inflated. As the design uses paternal half-sibs, it not only eliminates a nonadditive genetic effect — for example, dominance effect due to interaction between allelic forms of one gene at the same locus — but also DNA mitochondrial maternal effects. In addition, some environmental effects causing resemblance are eliminated. This enables us to estimate the effects of common environment on half-sib groups. As this is the first study of its type, we hope that it will provide a sound empirical and theoretical basis for research in cognitive traits. I must, however, emphasize that, given the design of the project, only limited inferences can be made from it.

Butler notes that the author of the report in *L'Express* acknowledges "that his perspective is coloured by his vociferous opposition to the genetic bases of human behaviour". The excesses of some behavioural geneticists are well known. I have myself drawn the attention of the scientific community to the weaknesses of their research²⁻⁵. Using poor data, on the basis of the model fitting they make exaggerated claims. Our study provides data not only on 103 children born by AID but also on certain traits of more than 3,000 other French children. Some researchers may regard the availability of reliable data on these children as a contribution to scientific knowledge.

The ethical problems posed involve the philosophical values of the scientific community as well as those of the community at large. These need to be discussed and debated, but in an informed way. The scientific content of research must remain in the domain of scientists but the community has the right and indeed the duty to set the parameters in which this research takes place.

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Does drug use cause AIDS?

M. S. Ascher, H. W. Sheppard, W. Winkelstein Jr & E. Vittinghoff

A hypothesis identifying substance abuse as a main cause of AIDS has naturally excited much publicity. But such claims have no basis in fact.

SINCE 1987, Peter H. Duesberg, a professor of molecular biology at the University of California, Berkeley, has maintained that the human immunodeficiency virus (HIV) is not the infectious aetiological agent for AIDS (acquired immune deficiency syndrome)¹. Responses to Duesberg have presented a strong case that HIV has a central role in AIDS pathogenesis^{2,3}. Duesberg did not at first present a clear alternative hypothesis, but recently, based on an ecological analysis of drug use in the United States, he has proposed that "either drug consumption (frequently associated with malnutrition) by recently established behavioral groups or conventional clinical deficiencies and their treatments are necessary and sufficient to cause indicator diseases of AIDS"^{4,5}. Because of the wide publicity attracted by this assertion, we decided to assess this hypothesis.

In the first case reports of AIDS in the United States, recreational drug use was considered as an aetiological factor⁶. Subsequently, an association between drug use, acquisition of HIV and development of AIDS has been extensively documented among those sharing needles for injecting drugs⁷. But the first major case-control study of AIDS in homosexual men showed no association between drug use and the development of AIDS⁸. Later, Kaslow and colleagues analysed data from more than 4,500 homosexual/bisexual men in the Multicenter AIDS Cohort Study (MACS) and found no association between the use of alcohol or other psychoactive drugs and HIV seroconversion or progression of HIV infection to AIDS⁹. Because amyl nitrite inhalants were thought to be aetiotically related to Kaposi's sarcoma¹⁰, Lifson and colleagues compared the use of 10 psychoactive drugs in 72 incident cases of Kaposi's sarcoma with 109 incident AIDS cases without Kaposi's sarcoma from the well-characterized San Francisco City Clinic Cohort, and found no differences¹¹. The most commonly used drugs were marijuana, nitrite inhalants, cocaine and amphetamines.

Direct test

To test Duesberg's drug-use hypothesis directly, we analysed data from a unique population-based cohort study, the San Francisco Men's Health Study

(SFMHS)¹². This study is based on a cohort of 1,034 single men 25–54 years of age at the time of recruitment (June–December 1984), selected by stratified, random, household sampling from neighbourhoods of San Francisco where the AIDS epidemic had been most intense before 1984. Participants were recruited without regard to sexual preference, lifestyle or HIV serostatus (not known at the time), and thus constitute a representative cross-section of men in this community. We believe that this sampling method is critical in the assessment of drug-use data, which can be strongly affected by recruitment bias.

Duesberg has stated⁵ that there are no controlled studies of drug use and AIDS, and that previous studies "failed to match the HIV-free control group with the HIV positives for the extent and duration of drug consumption". We performed our study in part to respond to this criticism. We examined the cohort at 6-monthly intervals for 96 months, and obtained drug-use data and determined HIV serostatus at each examination. Data for the variables we considered were available from 1,027 study subjects.

We compared heavy drug use (weekly or more frequent use of the four recreational drugs mentioned above) for the 24-month period before entry into the study among 215 heterosexual and 812 homosexual/bisexual cohort members. Except for amyl nitrite, with 18% heavy use in homosexuals versus no heavy use among heterosexuals, the percentage of subjects reporting heavy use of each drug was similar in both sexual preference groups: 36 versus 39% for marijuana; 7 versus 4% for cocaine; and 1 versus 5% for amphetamines, respectively. During 96 months of follow-up, 215 cases of AIDS occurred among the homosexual/bisexual men (cumulative incidence, 26%) compared with none among the heterosexuals. If heavy use of marijuana, cocaine or amphetamines is

casually associated with AIDS, a cumulative incidence of 56 (0.26×215) cases among the heterosexual subjects would be expected.

Survey results

Table 1 summarizes the cumulative incidence of AIDS and total mortality according to sexual preference and HIV serostatus on entry into the SFMHS. Of the 215 heterosexual men, none was HIV seropositive on entry and one seroconverted during the follow-up period. Among these men, no cases of AIDS and one death (0.5%) were recorded. Among the 812 homosexual/bisexual study subjects, 367 were HIV seronegative on entry and have remained so for 96 months. No cases of AIDS and seven deaths (2%) have been recorded among these men. Forty-five men seroconverted during the 96-month follow-up period. Among the seroconverters, eleven cases of AIDS (24%) and five deaths (11%) have been recorded. Among the 400 study subjects who were HIV seropositive on entry and throughout the follow-up period, 204 (51%) have developed AIDS and 169 (42%) have died.

Because Duesberg has specifically implicated amyl nitrite in the aetiology of Kaposi's sarcoma, we performed additional analyses to assess this relationship. As can be seen in Table 2, among the 144 homosexual/bisexual men who reported heavy use of amyl nitrite inhalants during the 24 months before study entry, 54 developed AIDS during the ensuing 96 months (cumulative incidence, 37.5%). Among the 668 homosexual/bisexual men reporting none or less than weekly use of amyl nitrite inhalants during the same time period, 161 developed AIDS in 96 months (cumulative incidence, 24%) for a relative risk of 1.56. This crude association is apparently the basis for Duesberg's hypothesis. Further analysis of the data reveals a similar association between

TABLE 1 Incidence of AIDS and death according to sexual preference and HIV serostatus

Sexual preference	Serostatus	AIDS cases		Deaths	
		<i>n</i>	<i>n</i> %	<i>n</i>	%
Homo/bisexual	Negative	367	0 0.0	7	1.9
	Converted	45	11 24.4	5	11.1
	Positive	400	204 51.0	169	42.3
Heterosexual	Negative	214	0 0.0	1	0.5
	Converted	1	0 0.0	0	0.0
	Positive	0	0 0.0	0	0.0

TABLE 2 Effect of nitrite use on incidence of HIV, AIDS and Kaposi's sarcoma in homosexual/bisexual men

Nitrite Use	AIDS cases			HIV positives		AIDS in HIV+		AIDS in HIV-		KS cases		KS in HIV+		KS in HIV-	
	n	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Heavy	144	54	37.5	105	72.9	54	51.4	0	0.0	26	18.1	26	24.8	0	0.0
Light	668	161	24.1	340	50.9	161	47.4	0	0.0	66	9.9	66	19.4	0	0.0
Relative risk (conf. interval)			1.56 (1.21-2.01)		1.43 (1.26-1.63)		1.09 (0.87-1.36)		NA		1.83 (1.19-2.80)		1.28 (0.85-1.91)		NA

drug use and HIV positivity, and when controlled for HIV serostatus, there is no overall effect of drug use on AIDS. A similar effect, a marginal association that drops after controlling for HIV serostatus, is seen in cases which end in Kaposi's sarcoma. Thus, when proper methods are used to assess the role of confounding variables, there is no evidence of a drug effect. In addition, we have performed a logistic analysis of the longitudinal drug-use data which shows no positive association between long-term or continued drug use and the development of AIDS.

AIDS definition

The clinical case definition for AIDS has been criticized as having subjective features and low specificity. We have attempted to circumvent this problem by assessing the effects of drug use on a more objective and, indeed, the primary pathognomonic feature of AIDS, CD4⁺ T-lymphocyte depletion. The relationship between HIV serostatus, drug use during the 24 months before entry into the study, and CD4 cell levels is shown in the figure. A consistent loss of

CD4⁺ T lymphocytes is limited to HIV-seropositive subjects, among whom there is no discernible difference between the counts according to composite drug-use scores. The apparent flattening of the trajectories over time is due to the death of rapidly progressing HIV-seropositive subjects. Among HIV seronegatives, the CD4⁺ T-lymphocyte count trajectories are essentially flat with slight but consistent differences between the drug-use groups. By contrast with Duesberg's hypothesis, moderate or heavy drug users have consistently higher counts than non-users, for unknown reasons. Taken together, these data do not support Duesberg's hypothesis that drug use causes AIDS.

Duesberg has argued repeatedly that studies of the associations of HIV infection, drug use and AIDS have been "uncontrolled". However, the population-based SFMHS provides a rigorously controlled epidemiological model for the evaluation of aetiological hypotheses. Duesberg has also argued that the case definition of AIDS is "circular" and that HIV-seronegative AIDS cases are excluded by definition. In the

SFMHS, AIDS cases were diagnosed by the clinical criteria defined by the Centers for Disease Control (CDC), which are irrespective of HIV infection status. Incidentally, the CDC's criteria for defining AIDS do not require HIV positivity, provided that the CD4 count is less than 400 and the clinically defining conditions are not explainable by established aetiologies¹³. Furthermore, in the data presented here, mortality as well as AIDS incidence support the HIV aetiological hypothesis.

Conclusions

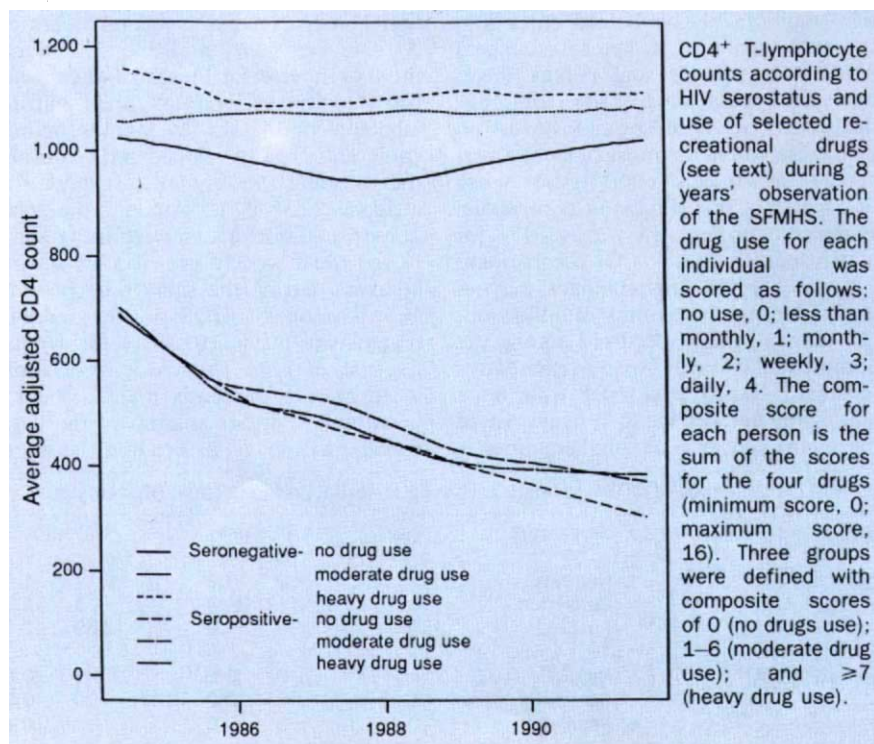
The main purpose of the cohort studies conducted in San Francisco and elsewhere has been to look for associations of environmental or behavioural factors with the development of AIDS. Had any factor other than HIV infection been found, it would have been reported immediately. The fact that not every failure to find such associations has been reported does not undermine the well-established causal relationship between HIV and AIDS, particularly as it relates to prevention strategies.

The energies of Duesberg and his followers could better be applied to unravelling the enigmatic mechanism of the HIV pathogenesis of AIDS. To this end, we have proposed an alternative model^{14,15} based on HIV signalling at CD4 cells. This model and others are now being evaluated, and we cordially invite Duesberg to participate in this endeavour. □

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Watson, Crick and the future of DNA

A season of celebrations of the fortieth anniversary of DNA appropriately began last week at Watson's laboratory on Long Island. Rarely do people who have worked revolutions have the luck to see the consequences.

Cold Spring Harbor. This research campus was modestly *en fête* last week, celebrating a little early the fortieth anniversary of the structure of DNA. But the key-word was "modesty". Both the principals were modesty itself, musing on their naivety all those years ago. "We got that wrong", Crick would say. What could ever have persuaded Watson to begin *The Double Helix* with the line that caused such offence, "I had never known Frances Crick in a modest mood"? It cannot just be age; immodest old men abound. Maybe it helps to know that you have changed the world.

Crick set the tone with a talk of the kind in which he specializes: not a wasted word. One of his criticisms of the film *Life Story* was that the script-writer had not captured "the style in which I talk". Unsurprisingly, he has aged. He stoops slightly, although not noticeably more than a tall man must to make eye-contact with his interlocutors. His hair, quite white, is cut to the level of his collar.

The talk was deft, as deft as a double helix with the strands running in opposite directions. What was it like then? No rushing to the lab. No secretaries, but no mail to speak of either. No travelling, except perhaps to Paris. No trans-Atlantic telephone. Hardly any literature to read. And no keys to the lab at night (so that people had to climb the railings if they wanted to work then). Much was done in pubs.

This is the legend of English academic life. Crick's purpose — but he is too much a gentleman to have declared it — may have been dangerously nostalgic: to demonstrate by example that it is possible to do good science without being solemn, even that science can be good fun. He laughed a lot, mostly at himself.

Of most others, he spoke well. Bernal was given credit for working out how to take X-ray pictures of protein crystals without them dehydrating. Rosalind Franklin (whose helical patterns of spots Watson saw on a famous visit to King's College, London) might have made faster progress if she had done a little model-building. And Pauling, in *The Double Helix* the man whom the pair felt breathing down their necks, "has not been given the credit he deserves" for laying the foundations of molecular biology (with the α -helix) and, more generally, for having preached that this part of biology is but a branch of physical chemistry.

So why has molecular biology prospered on the back of a succession of techniques that seem to come along just when they are needed — restriction enzymes, recombinant

DNA, ribozymes and so on? Because people have learned to let biology do the work for them. Purist chemists may think that the presence of an enzyme corrupts an experiment, but molecular biologists know better. Already, some have used "natural selection in a test-tube" to select molecules that do particular jobs: that will be a "technique for the future".

Modest to the end, Crick declared that "what Jim and I did was to accelerate by perhaps a few years" what would have happened anyway. And while there have been nucleic acids on the surface of the Earth for some billions of years, only in the past half-century have people understood why they are important. What, the unspoken question was, does that imply?

After that, if the hundred or so participants had been told that it were time for them to go, they would not have felt cheated. But they were not. And they did not. And most other nostalgia was ruled out. True, Paul Doty, speaking for the handful of those who knew in 1953 that that year would be a turning-point, conveyed the sense that people then had (in the context of DNA as a macromolecule) that they were working in the dark, but were certain soon to come across a blinding light.

That, the first of many blinding lights, was the discovery of messenger RNA. Crick's explanation of what seemed like the five-year hiatus after 1953 is that the expectation that the messengers would be RNA was confounded by the huge dominance of ribosomes as the places where RNA is sequestered in every cell. "Who would have thought that the messenger molecules would have been just one or two per cent of the total?"

The next light was the technique for making recombinant DNA, described by Stanley W. Cohen of Stanford, partner with Herbert Boyer in the business of joining pieces of DNA together. Crick says that he has been more surprised by that development than by anything else in the past forty years. Richard Roberts from New England Biolabs had earlier explained how the roster of sequences that can be cut by restriction enzymes almost filling up, and that it is only a matter of time before those that cannot be extracted from bacteria are synthesized as ribozymes or otherwise.

What is it all for? The richest diet followed: Wally Gilbert on the benefits of sequencing DNA, Mark Ptashne on gene control, Peter Dervan on how to fit third strands into the major groove of the double

helix, Tom Caskey on diagnostic tools based on DNA, Sol (as he is called) Snyder on gaseous neurotransmitters (there is now CO as well as NO) as well as talks on improving on nature's proteins and plants (insecticide genes can actually be transplanted) and the use of animals as biochemical factories. French Anderson, with three fresh approvals from NIH's Recombinant DNA Committee (RAC) in his pocket, gave the most robust public answer yet to the inevitable ethical question: sure, germ-line therapy will come, but nobody in his senses would do that on the evidence of fewer than a hundred patients now being treated in one way or another.

The oddest talk was by Kary Mullis, the inventor for Cetus Corp. of the technique of replicating *ad libitum* fragments of DNA and RNA. He spoke like one who had read *The Double Helix* and modelled himself on the author's account of his youth; there was more about his wives (two) and girlfriends (one of whom was present) than about PCR. Plainly he is a very clever man (and this very year a winner of the Japan Prize); his friends no doubt wish him well in his plan to sell copies of pop-stars' genomes. But he is also ageing at the same rate as other people. Crick claims to be still twelve years older than Jim, and "Jim", as he is called by everybody, is a loose cannon to be reckoned with.

It is curious, last week's participants may have reflected, how some things do not change with time. Crick (according to *The Double Helix*) was the cautious one, Watson the mercurial fellow oscillating between enthusiasm and doubt. It is still like that. Jim's counterpart of Crick's talk was a homily on the importance of being one's own man, of not putting up with other people's ideas of what the world is like.

That conviction is one reason why he has been such a success as director of this laboratory campus. But some things rankle. As the *New York Times* would say, his closing remark that if you feel your colleagues are against you, "quit before they fire you" was "understood by informed sources" to be a reference to his difficulties last year with Dr Bernadine Healy (who has resigned as director of the US National Institutes of Health).

Ten years from now, there will be even more impressive celebrations of the half-century of DNA. On present form, there is every chance that the same principals will be there. Meanwhile, it is good to see that they have become good friends again. Modesty apart, they did change the world.

John Maddox

Sickly channels in mild disease

Christopher Miller

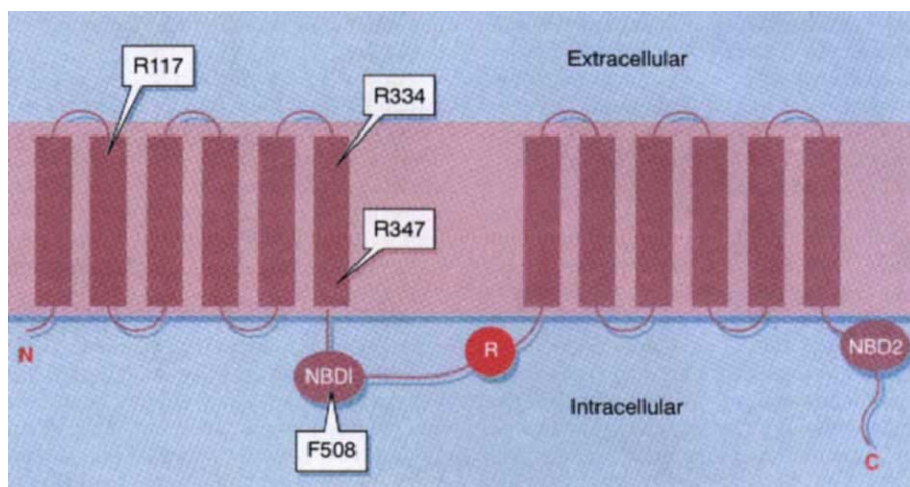
FROM the intellectual viewpoint, cystic fibrosis (CF) rivals sickle-cell anaemia as the prime example of a heuristically rich molecular disease — it can be viewed all the way from bottom to top, from the primary error in DNA sequence to the clinical manifestations. The genetic error results in defective epithelial Cl^- ion channels essential to fluid secretion, and a small army of researchers is at work on identifying just what the functional consequences are for the channel of naturally occurring mutations of the CF gene. On page 160 of this issue¹, Sheppard and colleagues present such a sequence-to-function mapping for several of the gene's alleles.

Our molecular understanding of CF, a devastating disease, has emerged with amazing speed. Only five years ago the causative lesion was unknown, even at the level of cell physiology, although defective epithelial transport of Cl^- was widely suspected to be the culprit. Then, in 1989, the altered gene was cloned^{2,3} and its product named the cystic fibrosis transmembrane regulator (CFTR) because of its 12 putative membrane-spanning α -helical stretches (see figure). A robust debate immediately ensued about the precise function of the CFTR. Is it indeed an epithelial Cl^- channel, or perhaps a protein involved in the delivery of Cl^- channels to the plasma membrane? The question now seems settled: CFTR is a Cl^- channel. This assertion would have elicited uproar only a year ago, but now two lines of evidence make it hard to avoid. First, certain point mutations in the CFTR gene (transfected into mammalian cells and assayed by patch recording) alter the interanionic selectivity of the open channel without affecting the regulation of its gating by phosphorylation and ATP⁴. Second, the CFTR protein, heterologously expressed and purified (in the denaturing detergent SDS, no less), induces Cl^- channels with the correct properties when reconstituted into 'artificial' planar lipid bilayer membranes⁵.

These electrophysiological assays set the stage for a detailed examination of the behaviour of channels arising in commonly occurring CFTR mutants. About 70% of CF patients carry the same mutation, a single amino-acid deletion (ΔF508) in one of the two nucleotide-binding domains (see figure). This mutation results in a temperature-sensitive defect in protein processing (not aberrant regulation by ATP, as might have been expected); at 27 °C, the mutant CFTR forms functionally active channels, but at 37 °C, it gets stuck in

the maturation pathway and fails to reach the plasma membrane⁶, thus giving rise to a very severe disease state in which the epithelial Cl^- channels are absent.

The report on page 160 examines three less common mutations (affecting about 2% of CF patients) that lead to mild forms of the disease. Each of these missense mutations alters a different arginine residue, one in the second



Positions of the mutations in the CFTR protein discussed in the text. NBD1 and NBD2, nucleotide-binding domains. R, regulatory phosphorylation domain.

transmembrane helix (M2), and two in the sixth (M6). When CFTR complementary DNAs bearing these individual mutations are transfected into mammalian epithelial cells, appropriately regulated Cl^- channels appear in the plasma membrane. However, the absolute amount of Cl^- current per cell is abnormally low, 5–30% of that induced by wild-type CFTR. Single-channel analysis revealed the reasons for this diminished whole-cell current. The two charge-neutralized M6 mutants, R334W and R347P, show a lowered single-channel conductance implying an altered Cl^- conduction pathway. The M2 mutant R117H, activated as usual by phosphorylation, displays a unitary conductance only slightly lower than normal, but its probability of being open is vastly reduced.

These different types of alteration in single-channel properties nicely account for the low whole-cell currents seen in the mutants, and they rationalize the mild nature of the disease in which the patient retains significant Cl^- transport function. The results also focus mechanistic attention on M2 and M6, highlighting how nature has already constructed point mutations affecting both gating and open-channel conduction properties.

More than 300 CFTR mutant alleles have been identified by genetic screening so far, and they provide a fertile field, already under cultivation, from which to harvest insight into the molecular mechanism of this crucial ion channel.

Cystic fibrosis is prevalent in Europe and North America, about 5% of Caucasians being heterozygous for deleterious forms of the gene. An obvious question arising from the disease aetiology is why the clinically severest mutant CFTR gene — effectively a null phenotype — is so much more prevalent in the population than the less harmful variants. Why, indeed, has the ΔF508 mutation spread

so quickly through the population in less than 10,000 years⁷? Questions such as this invite speculation as to the possible selective advantage conferred upon CF-heterozygous individuals, and the field is replete with untested guesses⁷. My favourite, offered by Baxter *et al.*⁸, is that a deficiency in epithelial fluid secretion (perhaps subtly present in heterozygotes) would ameliorate the severity of enterotoxin-elicited diarrhoea, which is by far the most important cause of infant mortality in primitive societies — susceptibility to lethal dehydration has probably been with our species throughout our evolutionary history and could account for a protective effect of this recent mutation. □

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Advent of a new family

Richard Aldrich

WITH the publication of sequence after sequence for potassium channels, many in the field began to believe that, with the exception of the embarrassing uncle minK, all such channels would turn out to belong to the same family. Not so, it seems. Two papers, one published last week¹ and one on page 127 of this issue², show that a major group of potassium channels, the inward rectifiers, come from a different, distantly related family.

Inward rectifiers are characterized by a greater tendency to allow potassium to flow into the cell rather than out of it. They are common in many cell types and have been studied electrophysiologically to a great extent³. The rectifying behaviour comes from a voltage-dependent block by internal magnesium, an intrinsic voltage dependence of gating with the channels opening upon hyperpolarization, or both. An important property is that the range of voltages over which the channel is open depends upon the external potassium concentration; as external potassium is raised, the voltage range of channel opening shifts to more positive voltages. Because its properties are so different from the voltage-gated channels that have received so much molecular attention, cloning and characterization of an inward rectifier gene has been the goal of a number of laboratories.

Body plan

The first hints of success came last December, when Schachtman *et al.*⁴ described the expression, in *Xenopus* oocytes, of a putative potassium channel cloned from the plant *Arabidopsis* (KAT1). The channel displayed a strong inward rectification, being activated only when the membrane was hyperpolarized to about -120 millivolts. These currents were blocked by caesium and tetraethylammonium, but it was not determined whether the range of voltage-dependence depended on external potassium concentration. These findings, along with the report that KAT1, and a related *Arabidopsis* clone AKT1, had hydrophobicity profiles similar to the familiar six putative membrane-spanning regions plus pore region of Shaker family potassium channels^{5,6}, led to some tentative conclusions and questions. First, it seemed that potassium channels from functionally quite distinct classes (even those from plants) had the same basic 'body plan'. Second, it seemed puzzling to many that a hyperpolarization-activated KAT1 channel would have a similar putative voltage-sensing region (the S4) to depolarization-activated Shaker-like channels.

Third, because of the very hyperpolarized voltages required for opening the KAT1 channel, it was unclear what relation it had to the classic inward rectifiers studied in animal preparations.

Like all notable papers, the two new ones address existing issues in interesting ways and raise new ones. Both groups relied upon expression cloning in *Xenopus* oocytes, again demonstrating the usefulness of this approach. The two clones, ROMK1 from rat kidney cells¹ and IRK1 from a mouse macrophage cell line², are much smaller than Shaker-like channels, having 391 and 428 amino acids respectively. The most intriguing feature of the sequence is the high degree of amino-acid conservation in a region corresponding to the H5 or P region of Shaker channels that has been strongly implicated in potassium permeability and ion selectivity. Outside this region, sequence comparisons with Shaker channels reveal only limited similarity. Furthermore, hydrophobicity plots suggest that there are only two additional membrane-spanning regions, quite unlike the six found in Shaker family channels.

Both papers suggest that the body plan consists of a potassium-selective pore flanked by two membrane-spanning regions and large cytoplasmic amino-terminal and carboxy-terminal domains. Because of biophysical similarities of permeation between inward rectifiers and Shaker-like channels, the conservation of pore sequences, and the tetrameric nature of Shaker-type channels⁷, it seems reasonable to believe that these inward rectifier channels also function as homotetramers in the oocyte membrane (though no evidence specifically addressing this point has been published). The expression patterns of the two new clones are distinct — northern blots show ROMK1 expression in kidney and other tissues, but only weak or no expression in brain, heart and skeletal muscle; IRK1 is strongly expressed in brain, heart and skeletal muscle, but not in kidney.

The ROMK1 and IRK1 channels expressed in oocytes share a number of characteristics. They are both inward rectifiers, although IRK1 seems to rectify more, allowing considerably less outward current to pass. Both are blocked by barium, but IRK1 seems to be more sensitive. In addition, IRK1 shows dependence of opening on external potassium and highly voltage-dependent block by caesium; both are characteristics expected of inward-rectifier channels.

What, though, is the mechanism of

rectification? Although many inward rectifiers are blocked by internal magnesium, gating of some of them is also intrinsically voltage-dependent in that the probability of opening increases with hyperpolarization³. Experiments on magnesium block of ROMK1 or IRK1 channels is not presented by the two groups because of a tendency of these channels to run down or disappear in inside-out patches. The rundown of ROMK1 can be delayed by application of Mg-ATP to the internal face of the patches (this ATP-dependent regulation of rundown leads to the reference to ATP regulation in one of the titles¹, but it should not be confused with the widely studied ATP-sensitive channels which are turned off by intracellular ATP). The ability to increase channel lifetime in inside-out patches should make experiments on rectification by internal magnesium block possible.

Voltage dependence

As to intrinsic voltage-dependence as a mechanism for inward rectification, both groups report a decrease in open probability with hyperpolarization, opposite to that of some inward rectifiers⁸. In the present studies, the inward rectification appears to be instantaneous, with no increase in probability occurring during a hyperpolarizing voltage step. This is also unlike many native inward rectifiers, but could result from the slow time scale of the records obscuring an activation phase of the current. Further experiments with faster recording will allow closer comparison with previous studies.

An intriguing property of inward-rectifier channels is their apparent triple-barrel structure. With low magnesium concentrations, three distinct blocked states can be observed, and this is most easily explained by three ion-conducting pathways being gated together but being independently susceptible to block by magnesium⁹. This poses a puzzle about the structural basis of these parallel pathways. If the pore is constructed as is generally thought, with the H5 region from each subunit contributing symmetrically to its lining¹⁰, then does an inward rectifier consist of three homotetramers linked together in some way and gated together? Or can the three-barrel nature

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be accommodated by a single tetramer? This also brings up the general question of other subunits that may alter the function or be necessary for some essential properties. The only evidence on the possible role of other subunits is that both ROMK1 and IRK1 display similar properties when expressed as single clones in oocytes, as identified from expression of total messenger RNA from the original tissues. Such similarity suggests that other subunits are not necessary for the properties of the currents seen with total mRNA.

There are several aspects to the significance of these new channel clones. They identify a hitherto unknown family of potassium channels and allow detailed mechanistic work on their properties. They further substantiate the importance

of sequences in the H5 region for potassium permeation and selectivity. And they raise interesting questions about the nature of KAT1-like channels. Do these channels represent another class of inward rectifiers with a greater degree of intrinsic voltage-dependence of gating, thus making them more closely related to the voltage-gated Shaker-like channels? If so how do they achieve activation by hyperpolarization? The answers to these questions and other family secrets will no doubt be revealed shortly. □

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OCEANOGRAPHY

Green sea, black mud

Constance Sancetta

To the occupants of a space shuttle ten million years ago, the geography of the Earth would have looked only slightly different from its modern form. The Mediterranean was larger, and portions of southern Europe were submerged beneath shallow seaways, producing a somewhat miscellaneous grouping of islands and peninsulas. The Alps and Himalayas were lower, and had considerably less snow cover. In the eastern equatorial Pacific, there was no junction between North and South America; the Isthmus of Panama did not exist, and the Pacific and Atlantic oceans mingled their waters through a tropical seaway. So much has been known for decades.

We now learn that an instrument such as the Coastal Zone Color Scanner would have revealed a further anomaly: vast areas of this region were apparently covered by floating mats of diatom algae. The mats, each several tens of centimetres square, were probably grouped into massive patches by wind action, with the total area affected being the size of Australia. On page 141 of this issue, Kemp and Baldauf¹ report that this occurred repeatedly, and probably periodically, between fifteen million and about four and a half million years ago.

Basing their conclusions on a series of sediment cores recovered by the Ocean Drilling Program, the authors report a hierarchical clustering of mat-producing events, each alternating with conditions more like those of the present. The clustering runs from annual events, producing individual millimetre-scale laminae, to decimetre-scale beds representing periods of hundreds to a few thousand years. The latter can be corre-

lated between sites 2,000 miles apart, implying that for prolonged periods, conditions throughout the region were favourable for mat production.

Such mats are not unknown in the modern world, as the authors point out. They can be formed by several genera of diatoms, all characterized by an elongate cell geometry. It is the spatial extent and the repetitive nature of the sediment mats that render them so fascinating. The conditions under which mats occur are not well understood; it may be a combination of cellular positive buoyancy and calm sea-surface conditions which permits them rise to the surface². Once there, they may persist for weeks. Although the growth rate of the cells may not be particularly high, the sheer number and spatial extent of the mats results in a marked reduction of nutrients and production of biomass. When the cells lose buoyancy, the mats settle rapidly to the sea floor, carrying large quantities of pristine organic matter to the sediments³.

Kemp and Baldauf make a second important point here. The laminated sediments were formed under reducing conditions, so that the organic matter was not oxidized and remained at relatively high concentrations. Such sediments, termed sapropels (from Greek *sapros*, decay, and *pelos*, mud) are often found in settings where high organic input occurs in a semi-enclosed basin with limited circulation, leading to stagnant bottom waters with little or no dissolved oxygen. The most famous example is the Black Sea. So frequent is this association that it has come to be axiomatic among sedimentologists that

sapropelic sediments automatically imply anoxic bottom waters. As Kemp and Baldauf indicate, this need not be true. The overlying water column may be well oxygenated, provided that the sediment itself is reducing and that diffusion between water and sediment is limited. In the case of the diatom mats, the authors suggest that the dense meshwork prevented burrowing organisms from penetrating the sediments, thus limiting oxygen exchange.

A similar explanation may be true for the Pleistocene sapropels of the eastern Mediterranean, some of which also consist of diatom-mat laminae⁴. Some workers have called on conditions in which strong contrasts in salinity, possibly resulting from Nile flooding⁵, produced density stratification sufficient to limit deep water exchange through the entire region, thus supposedly producing anoxic bottom waters. In light of the findings in the eastern Pacific, it seems equally likely that existence of a stable surface water layer supported the production of mat-forming diatoms, whose deposition on the sea floor produced organic-rich sediment laminae below an oxygenated water column. At the least, we should re-examine the assumption of anoxic bottom waters wherever sapropels occur.

An intriguing point is that the mat production ceased about 4.4 million years ago. Kemp and Baldauf suggest a reduction in nutrient availability as the cause, but do not speculate on the conditions leading to such a reduction. Geochemical evidence has been used to infer that the gradual closure of the Isthmus of Panama resulted in a measurable differentiation of surface Atlantic and Pacific waters as far back as 4.0 million years ago (ref. 6). Possibly the increasing restriction of the seaway, both vertical and lateral, altered the water circulation in the eastern equatorial Pacific so as to affect either the nutrient supply or the physical conditions favouring mat formation.

In the late Miocene, there was no peak in Darien, and certainly no Spanish explorers. But if, by some miracle, Vasco de Balboa had been allowed to survey this vast sea, with its mats of algal green heaving gently upon the long-rolling waves, he might still have named it l'Oceano Pacifico, the peaceful ocean. □

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What's on the plume channel?

Ken Rubin and John Mahoney

MOST of the ocean floor is composed of basalt formed at mid-ocean ridges, where parting plates decompress the underlying asthenosphere, allowing it to rise and melt. Basalts are also erupted at hotspot volcanoes, situated over hot plumes of material probably rising from deeper within the mantle. Chemical differences between the resulting magmas reflect, in part, the different conditions under which they were formed; but they also reflect differences in the starting materials, and so are a sign of compositional variations in the mantle. Where plumes and mid-ocean ridges occur close together, the complexity becomes greater. Helium and other isotopes in basalts from such sites are providing insights into the processes at work, as was demonstrated at a recent meeting*.

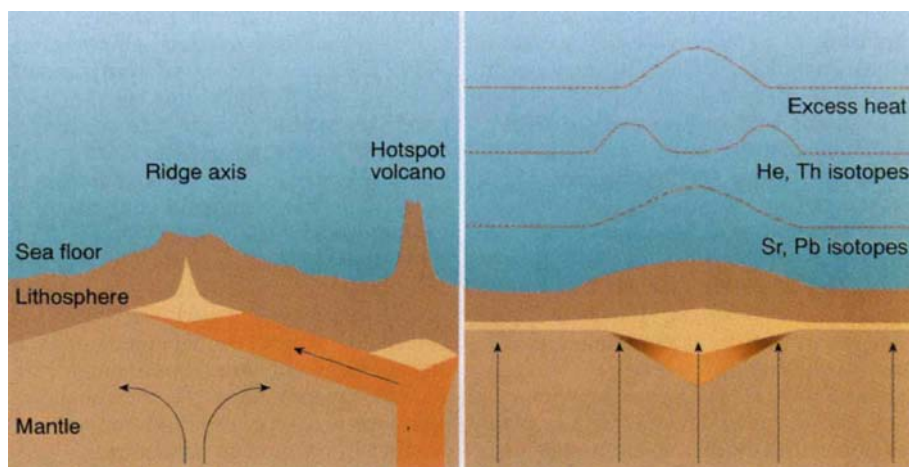
To a first approximation, most mid-ocean ridge basalts (MORBs) are compositionally alike, which has been taken to indicate that much of the asthenosphere is relatively homogeneous. Where differences have arisen¹, hotspot volcanism is often found in the vicinity, and mixing of the two sources is thought to account for the chemical anomalies. Iceland is the best known example of a near-ridge hotspot, and weakening anomalies along the Mid-Atlantic Ridge away from Iceland are in keeping with this picture: the plume rises to the ridge as a single jet and disperses along it². Elsewhere, hotspots as far as 1,000 km from a ridge have been found to contaminate MORB^{1,3,4}. The extent of the anomaly depends on the size of the hotspot and on its distance from the ridge axis^{1,5}. It has been suggested^{1,3,5,6} that where a ridge has passed over a plume, a channel forms at the base of the lithosphere through which the plume material continues to flow (see figure): where ridges are close to, but have never overlain, a hotspot (the Mid-Atlantic Ridge near Fernando de Noronha is an example), geochemical anomalies are conspicuously lacking.

Preliminary geophysical support for this plume-jet model was presented at the recent meeting. Seismic data (secondary-wave velocity profiles) from some near- and off-axis hotspots are consistent with the presence of a lateral channel of hot plume material extending towards the ridge axis (T. Tanimoto, University of California, Santa Barbara); the depth of the channel bottom is less for faster-spreading ridges. And gravity anomalies over the ridge axis in the Galapagos region are also consistent

with this notion (G. Ito, Woods Hole Oceanographic Institution).

A particular puzzle discussed at the meeting concerned helium isotope compositions. Normal MORB has a very constant $^3\text{He}/^4\text{He}$ ratio, but the ratio varies greatly in hotspot lavas: it is far higher than the MORB ratio at Hawaii and Iceland, for example, but far lower

volatiles at near-solidus temperatures and the extraction of small-degree silicate melts³) may be possible from measurements of several process-specific geochemical tracers. In the case of the East Pacific Rise anomaly associated with the Easter/Sala y Gomez plume, one of us (K. R.) has found that lavas with plume-like Sr and Pb isotopes and MORB-like $^3\text{He}/^4\text{He}$, also have extremely depleted $^{230}\text{Th}/^{232}\text{Th}$, Th/U, La/Sm and K/P compositions. These indicate processes that have modified the source material, but too recently to affect the



Idealized cross-sections of a fast-spreading mid-ocean ridge in the vicinity of an off-axis hotspot (after ref. 5). Left, across axis view, with the plume-jet (orange) flowing beneath the lithosphere towards the region of decompressional melting (yellow) beneath the ridge. Right, view from the side, with degrees of modification to the plume material indicated by graded shading: the least modified (darkest) is near the cooler edges of the plume (entrainment of asthenospheric material in the very wings causes some alteration). The modifications, arising before or as the lateral jet forms, or during its flow to the ridge, are the cause of the along-axis physical and geochemical profiles shown.

at St Helena, Gough and Tristan de Cunha. Where plume-type helium signatures are recorded along ridge axes, the peak values are often not found in the same places as the peaks for other isotopic tracers of plume material (ref. 3; B. Hanan, San Diego State University; R. Poreda, University of Rochester): thus, lavas with strong plume-like strontium, lead and neodymium isotope signatures but MORB-like helium isotope ratios may be found at one place along a ridge axis, and the reverse may be found at another. On the Central Indian Ridge near the Réunion hotspot, the $^{87}\text{Sr}/^{86}\text{Sr}$ plume signature is pronounced, but there is no helium indicator at all⁴.

Somehow, it appears, the helium isotope systematics are decoupled from those of Sr, Pb and Nd. Accepting the plume-jet model, the curious helium ratios are most probably caused by physical processes occurring within the mantle as the plume-derived material migrates to the melting zone below the ridge axis, rather than being attributable to variations in the source material itself.

Determining just what these processes are (likely candidates are degassing of

longer-lived Sr or Pb isotopes. Because only He would be affected if volatile degassing were the cause of the isotopic shifts, the combined data suggest that a localized small-scale melting event (or events) preceded melting beneath the ridge axis; this may have taken place when the plume material was passing through the vicinity of Easter or Sala y Gomez Island. Whether this process occurred many times or only occasionally is impossible to determine without further data on older MORB from the region.

There are also instances where enriched material is erupted at ridge crests in the absence of any obvious hotspot. For example, at about 17° S on the East Pacific Rise, enriched material occurs in discrete peaks of Pb, Sr, Nd and He isotopes along-axis, but the peak in $^3\text{He}/^4\text{He}$ is narrower and found slightly north of the others (J. M.). Although no hotspot is involved, degassing or small-degree melting during movement of enriched mantle material may again be at work, in this case as material moves into the sub-axial melting zone with a component of along-axis motion.

* American Geophysical Union Fall Meeting (session on Plume-Ridge Interactions), San Francisco, 7-11 Dec. 1992.

The idea that the mantle can be modified shortly before large-scale melting is not new, but only recently has the evidence come together to show how. That modifications may occur in shallow, laterally moving mantle emphasizes that variations in MORB in the most highly incompatible elements (such as Ba, Rb, Th and K), and at least some isotopic ratios, can only partly be ascribed to long-term variations in mantle source compositions. The exact nature of process-related modifications may vary with location, so evidence from multiple process-specific tracers (radioactive isotopes, volatiles) will become increasingly important as detailed case-by-case studies of individual sections of the ocean ridge system are carried out.

In addition, if we are ever to understand the complexities of compositional variability within the mantle and how they manifest themselves in MORB, more information is required about the inherent heterogeneities in the asthenosphere and in plume material away from the influence of ridges. Particularly, to what extent may a given plume vary over time (as indicated, for instance, by secular variations in the isotopic compositions of Hawaiian lavas⁷)? If the composition varies both vertically and horizontally within a plume, then the material supplied to an adjacent ridge crest will vary with time. Moreover, the modifications to the moving plume material, where the pertinent processes take place, and how and where they are expressed in lavas on the ridge could change. Combined geochemical and geochronological studies of near- and on-axis lavas using different radioactive dating schemes should clarify matters; ²²⁶Ra–²³⁰Th, ²³⁰Th–²³⁸U and ⁴⁰Ar–³⁹Ar dating should be good for samples 0–8, 20–350 and over 70 thousand years old, respectively.

The true power of geochemistry comes from the most comprehensive data sets, based on the maximum possible number of tracers. Even with the tools available now, the prospects are good for understanding mantle variability and how it is modified. Therein lies the light at the end of the plume channel. □

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Back to the future

Gabrielle Walker

WHILE most climate modellers have their sights focused on the future, seeking to understand how our world will respond to the threat of global warming, many are also looking to the past — for answers to the same question. This was evident at a Royal Society Discussion meeting last month*. The climate of the Mesozoic era (65–248 million years ago), the focus of the meeting, was generally much warmer than it is today, with globally averaged surface temperatures 6–14 °C higher than at present; understanding the reasons for the warmer temperatures then might help us to predict how increasing emissions of greenhouse gases into the atmosphere will perturb the Earth's climate in the future.

This point was illustrated by Eric Barron (Pennsylvania State University), describing experiments using a general circulation model called GENESIS to determine which of the geographical arrangement of the continents (the ancient supercontinent, Gondwanaland, was just breaking up) and the atmospheric carbon dioxide concentration was more responsible for the warm global temperatures in the Cretaceous period 65–144 million years ago.

In 1984, Barron and Washington¹ used an earlier, much less sophisticated model to address the same question. Then, they concluded that the Cretaceous distribution of the continents was the primary cause, with higher atmospheric CO₂ concentrations playing a minor role. This seemed to suggest that the Cretaceous might not be a good analogy for a future greenhouse world. But the earlier model included some rather crude assumptions. In particular, it incorporated only mean annual solar heating, and there was no thermal inertia built into the model ocean. GENESIS, on the other hand, allows for seasonally varying solar insolation, and has a mixed-layer ocean. And the results tell a different story.

To their surprise, Barron *et al.* found that changing from present-day to Cretaceous geography produced a mean global cooling of about 0.2 °C. It was only when the CO₂ content of the Cretaceous atmosphere was increased to four times the present-day value that global surface temperatures increased by 5.5 °C. Thus, it seems that the Cretaceous was, after all, a 'greenhouse' epoch, and might be able to tell us something about future climate change.

One of the most important unknowns in the quest to predict global warming is the climate sensitivity — the increase in global mean surface temperature asso-

ciated with a doubling of atmospheric CO₂. Different climate models calculate different sensitivities, mainly because they parameterize features such as cloud cover in different ways. The sensitivity calculated by GENESIS, 2.3 °C, is more or less in the middle of the range of likely values put forward by the Intergovernmental Panel on Climate Change (IPCC) (1.5–4.5 °C; ref. 2).

But the results from this study allowed Barron to estimate the atmospheric CO₂ concentrations that would be required if the sensitivity of the atmosphere were at either extreme of the IPCC range. If the required Cretaceous temperature range is 6–12 °C, a climate sensitivity of 4.5 °C would indicate Cretaceous CO₂ levels 2.7–5.3 times the present concentration, in keeping with the 2–6 times present CO₂ estimated by Berner³ using a geochemical model. On the other hand, a climate sensitivity of 1.5 °C would require atmospheric CO₂ concentrations to be 8–16 times the present concentration, higher even than Cerling's estimate of 4–9 times present using evidence from Mesozoic palaeosols⁴. So, when compared to geological evidence for the composition of the Cretaceous atmosphere, the model results from the Cretaceous suggest that the climate sensitivity to doubling of atmospheric CO₂ is at the higher end of the IPCC range.

This conclusion illustrates another purpose of the meeting, bringing together modellers and the geologists who piece together past climate from rock types and fossils. By the second day of the meeting, representatives of both groups were swapping ideas and planning collaborations. Meanwhile, there are various improvements for Barron *et al.* to make to their model. André Berger (Université Catholique, Louvain) pointed out that the model should take account of the lower solar irradiance in Cretaceous times (although this would probably drive the required climate sensitivity to even higher values). Mark Chandler (NASA GISS) and John Mitchell (UK Meteorological Office) both raised questions about the role that ocean heat transport might have played in the Cretaceous. But the overall message remains: the past seems to be yielding clues about the future. □

Gabrielle Walker is an assistant editor of Nature.

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The omnipotent nucleosome

Ken van Holde

EVER since the nucleosome was discovered in the early 1970s, molecular biologists have been wondering what it does. A compact bundle of DNA wrapped about a histone octamer, it is the ubiquitous structural motif in eukaryotic chromatin. From the first, it was clear that nucleosomes would be effective in compacting DNA in the nucleus. But do they do more? Until recently, the most that could be guessed was that nucleosomes might act as general repressors of transcription. All the interesting and subtle things were done, it was thought, by transcription factors. That view has begun to change, and now we have two papers^{1,2} that suggest that nucleosomes have a much more active role in transcription. The papers are connected not only in topic but also in having an author in common, Alan Wolffe of the National Institutes of Health.

It had long been recognized that one way in which a nucleosome might repress transcription is by sitting, like the proverbial dog in the manger, on a promoter site, thereby blocking transcription factors or polymerases from access to the promoter. There are a number of clearly documented examples of this. A classic and well-studied case is the regulation of the genes for 5S RNA in *Xenopus*. One of the main factors required to initiate 5S RNA transcription is TFIID — also famous as the first zinc-finger protein to be recognized³. Last year, Hayes and Wolffe⁴ demonstrated that placement of a nucleosome on the 5S gene would block TFIID binding. However, the entire nucleo-

some core (a tetramer of H3 and H4 plus two dimers of H2A and H2B) seemed necessary for the full effect; binding could still be observed if the H3/H4 tetramer alone was present on the DNA.

In one of the new studies, Lee *et al.*¹ show that it is the amino-terminal regions of the histone molecules that hold the key to blocking TFIID binding. The function of these regions, present as unstructured 'tails' on the otherwise globular histones, has long been a puzzle. The puzzle is compounded by the fact that it is these regions that bear highly conserved sites for post-translational modification — especially acetylation^{5,6}. Lee *et al.* find that either cleaving off the tails with trypsin, or increasing their level of acetylation, allows TFIID free access to the nucleosomal DNA. The acetylation result is particularly exciting, for it has been known for years that acetylation is somehow associated with transcriptional activity, but the causal relationship has remained obscure (see refs 5 and 6 for review).

The data show that the nucleosome is not displaced by either of the treatments nor by the subsequent TFIID binding. Two quite different models might be proposed to explain this. The first derives from the observations in Michael Grunstein's laboratory⁷ that H4-terminal tails are required for some promoter activations in yeast, and would envisage a direct interaction of TFIID with acetylated tails. This model appears to be ruled out by experiments in which the tails are proteolytically removed. Two variations of a second class of model are shown in Fig. 1. Acetylation is presumed to release the tails from the DNA⁶, and thereby either simply to make the nucleosomal DNA accessible, or to induce a conformational change in the nucleosome which promotes TFIID binding. Neither of

those possibilities can be excluded at the moment.

It is too soon to say just how significant this particular effect of acetylation may be *in vivo*, because acetylation produces other specific changes in chromatin structure^{5,6}. But forging this connection between acetylation and transcriptional activation in itself constitutes a major step forward.

A quite different and equally provoca-

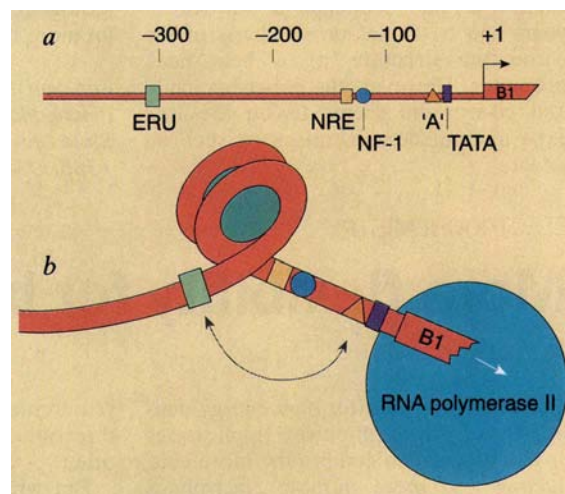


FIG. 2 A model for the chromatin organization of the vitellogenin B1 promoter. *a*, Representation of sequence, showing positions of the oestrogen responsive unit (ERU), and a complex of regulatory factor sites. These include binding sites for a negative regulatory factor (NRE), nuclear factor 1 (NF-1), and the 'A' factor site and the TATA box. *b*, The same structure with the positioned nucleosomes in place. (From ref. 2.)

finding comes from a collaboration between Wolffe and the group of Walter Wahli at the Institut de Biologie Animale in Lausanne (Schild *et al.*²). Again we are confronted with a nucleosome in a promoter region, this time in the *Xenopus* vitellogenin B1 promoter. Using *in vitro* chromatin reconstitution, Schild *et al.* have found that deposition of a nucleosome onto a site between 140 base pairs and 300 base pairs upstream from the transcription start site potentiates stimulation of transcription by oestrogen and oestrogen receptor. Deletion or even shortening of this sequence abolishes the stimulating effect of oestrogen; the nucleosome now covers the oestrogen response unit (centred at about -320). But why does the mere

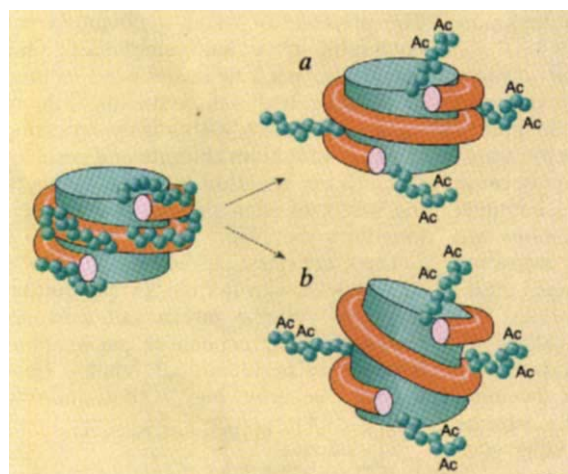


FIG. 1 Two models to explain the influence of histone acetylation on transcription factor binding. *a*, Acetylation releases DNA tails from nucleosomal DNA, allowing the factor access to the latter. *b*, Release of histone tails also causes a conformational change in the nucleosome, promoting binding of transcription factor. (Adapted from ref. 1.)

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presence of a nucleosome on the native sequence stimulate the response to oestrogen?

A scheme for what may be occurring is shown in Fig. 2. The oestrogen-receptor binding sites are far upstream from the complex of transcription-factor binding sites on the DNA sequence. But a nucleosome at the position found in this study will, by looping the DNA, bring these two groups of control elements close together. This model is quite similar to one proposed a number of years ago by Elgin⁸ on the basis of the chromatin structure of a heat-shock promoter. However, the paper by Schild and co-workers seems to be the first experimental demonstration of such an effect.

Again, this is a finding that may account for many previously puzzling observations. The considerable spacing on DNA sequence between supposedly interacting regulatory elements has been noted in a number of cases and never satisfactorily explained. Perhaps the most important lesson from both of these studies is that chromatin structure cannot be disregarded in attempts to understand eukaryotic transcription. That will make the lives of molecular biologists a bit more complicated but a lot more interesting. □

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ELECTROCHEMISTRY

More flexibility for batteries

Malcolm Ingram

LITHIUM batteries offer high energy densities, but for high-power applications or use in extreme conditions, more conducting and more durable electrolytes are needed. The work of Angell *et al.* on a new class of 'salt-in-polymer' electrolytes, reported on page 137 of this issue¹, raises the hope that these may be found. The authors dissolve poly(propylene oxide) or poly(ethylene oxide) in a low-melting-point 'cocktail' of lithium salts (reversing the usual strategy of dissolving the salt in the polymer). The result is a new class of rubbery materials, which are good Li⁺-ion conductors even at ambient temperatures. The incorporation of such electrolytes into high-energy, high-power density rechargeable lithium cells could widen the use of batteries in sensing and energy storage, and give a fresh impetus to the development of electric vehicles.

The search for new solid-state lithium batteries has until now been beset with difficulties. These relate both to the resistance of the electrolyte (when this is large, high-power applications are excluded) and to compositional and volume changes occurring within the cell during charge-discharge cycles: the lithium electrode shrinks during anodic dissolution, while intercalation of lithium into the counter-electrode (which may be of MnO₂ or TiS₂) causes a corresponding expansion.

Ideally, the electrolyte will conduct only by Li⁺ ions, entering at the anode and leaving at the cathode to preserve its composition; all volume changes occurring must be absorbed by an elastic deformation of the electrolyte. Generally, it has proved difficult to find a material which will satisfy both these

requirements. Indeed success in one direction tends to preclude success in the other.

But what kinds of lithium electrolyte are currently available? There are systems where lithium salts are dissolved in solvents such as propylene carbonate or thionyl chloride. But the leakage problem with liquids such as these is well recognized, and the volatility of such solvents introduces fire or explosion hazards if the batteries are overcharged, or used at excessive temperatures (say 150 °C). The choice is therefore between lithium-ion conducting ceramics (including ion-exchanged β -alumina²), lithium-ion conducting glasses^{3,4} and the elastomeric (or rubbery) polymer electrolytes⁵. In recent years, increasing attention has been devoted to the amorphous electrolytes.

Some ten years ago, Angell⁶ distinguished between the very different mechanisms of ion transport in glasses and polymers. He showed that ion transport in glasses is possible because cationic hopping processes continue although the structural relaxations are frozen in below the glass transition temperature, T_g . In the glasses, therefore, only the Li⁺ ion is mobile. This makes the glass electrochemically compatible with lithium electrodes, but there are problems associated with the inherent brittleness of glass and the extreme sensitivity of the more interesting glass compositions³ to moisture.

On the other hand, the migration of ions in polymer electrolytes is strongly coupled to the liquid or rubber-like (segmental) motions of the host polymer. The polymer electrolytes can only be used above T_g . This solves the brittle-

ness problem, but the disadvantage is that cations and anions are now both mobile.

A typical polymer electrolyte could be obtained by combining lithium perchlorate with poly(ethylene oxide). The driving force here for salt dissolution is the complexation of Li⁺ cations by the ether oxygens of the polymer. The expected result, namely that perchlorate ions are in fact more mobile than the tightly bound cations, has been verified experimentally⁵. The end result is that the composition of polymer electrolyte can change during the electrolysis, with an adverse effect on battery performance. An added problem is that the conductivity of polymer electrolytes is usually insufficient at room temperature (often less than 10⁻⁵ S cm⁻¹).

The significance of Angell's new work is that he appears to have found a way of combining the best features of glassy and polymer electrolytes. His trick has been to exploit the properties of low-melting-point molten salt mixtures. These mixtures, which may contain acetates, chlorates and perchlorates of lithium, and also some aluminium chloride, supercool to yield liquids that are highly conductive at room temperature. Angell claims that the high conductivity stems both from a small degree of decoupling (proximity to T_g) and from operating above T_g . In effect the melts are just near enough to T_g to exhibit a 'solid like' (Li⁺ ions only) mechanism. Dissolution of polymer into these melts produces a rubbery version of a 'glassy' electrolyte, which has long been a goal of researchers in this area.

Much work remains to be done before this discovery can be fully exploited. Angell himself points out the highly hygroscopic nature of melts containing lithium chlorate or aluminium chloride. The presence of water is obviously unfavourable to lithium electrodes. One would also need to recommend caution in building batteries containing lithium metal in contact with highly oxidizing mixtures of lithium chlorate and perchlorate. Close attention must be given to the safety of such devices and to their long-term shelf life.

These are questions which one expects can be dealt with in time. It may be that the most suitable molten-salt mixtures for such electrochemical applications have yet to be identified. What is clear is that by achieving a thousand-fold

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increase in Li^+ mobility at ambient temperatures compared with conventional 'salt-in-polymer' electrolytes, a way has been opened to a new generation of lithium batteries with high-power density applications in prospect.

Equally important now that such a rise in ionic mobility has been achieved without the use of volatile 'plasticizers' such as propylene carbonate, a class of materials emerges which may be ther-

mally stable up to 150° or even 200 °C. These electrolytes could therefore bridge the present gap in battery technology between essentially ambient-temperature devices which operate up to 100 °C and the high-temperature molten salt systems. □

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OPTICS

Turning on quantum taps

Stephen M. Barnett

MEASURING a quantum-mechanical observable should collapse the wavefunction so that all future measurements give the same answer, but in practice the act of observation generally demolishes the original quantum state of the system, leaving no possibility of future measurements. J. Ph. Poizat and P. Grangier (*Phys. Rev. Lett.* **70**, 271–274; 1993) have now circumvented this sticky problem by developing a method of delicately tapping off information on the state of a laser beam whilst leaving the signal unchanged.

Quantum mechanics is fundamentally a probabilistic theory. It is usually the case that we can predict only the probability that a measurement will yield a particular result. If we prepare a large number of copies of our system in the same state then we will find that the results of individual measurements will differ but that the distribution of results will be characteristic of the state prepared. A simple example is the measurement of one component of spin possessed by a spin-half particle. Individual measurements will always give the results $+\hbar/2$ or $-\hbar/2$, and the average over many observations will produce the average value of the spin component.

The ideal model of a measurement in quantum theory has two properties. First, it records one of the possible values of the measured observable; second, it changes the state of the system, so that a sequence of ideal measurements carried out on the same system will always produce the same result. For our spin-half particle this means that if one measurement of the z -component of the spin leads to the result $+\hbar/2$ then so will all subsequent measurements.

This theoretical ideal is rarely achieved in experiments, as the act of observation is usually too violent for the quantum state to survive intact. This is especially true in quantum optics. We can count the number of photons in a

beam with great accuracy, but the photodiodes used to count the photons also absorb and thus destroy them. We can certainly do the measurement but fall far short of the theoretical ideal of leaving the state with a precisely determined number of photons, ready for repeated observation.

To approach the ideal quantum measurement for light we require a less violent detection process. Suitable processes are referred to as quantum nondemolition (QND) measurements. With a QND detector for light we could measure any information encoded on the amplitude of a laser beam without degrading the quality of the information left on the beam. Such a detector could justly be dubbed a quantum optical tap, in that it allows us to tap into an optical signal and measure the amplitude modulation without changing the signal.

Poizat and Grangier's quantum optical tap works by the nonlinear optical effect of cross-phase modulation. In a suitable nonlinear medium (in this case a sodium cell) the refractive index will be modified by light propagating through it. The index change will be proportional to the intensity of the light, so it leads to an intensity-dependent phase modulation. It is this phase modulation that is responsible for the production of solitons (self-sustaining wave packets) in optical fibres. When two beams are present, the intensity-dependent modification of the refractive index induced by one beam can produce a modulation of the phase in the second beam. This cross-modulation means that the phase change of one beam is proportional to the intensity of the other, so by monitoring the phase of a 'meter' beam which has interacted with the signal beam we can infer the intensity of the signal.

As the signal is not absorbed, it continues to propagate and could in principle be measured again in the same way. Poizat and Grangier in fact measure the signal by conventional means after the QND measurement. Their comparison

of these two measurements shows that the signal does not become appreciably depleted, and that the signal and meter beams are correlated so that the measurement carried out on the meter tells us something about the signal amplitude. This new experimental arrangement has achieved the sensitivity required to push the nondemolition measurement into the quantum regime where the signal degradation is lower and the correlation between the meter and signal beams is stronger than could be achieved in any classical arrangement. A conventional measurement would either have to degrade the signal more to achieve the same degree of accuracy in the measurement, or sacrifice accuracy in order to leave the signal intact.

There is, of course, a price to be paid. Measurement of the amplitude scrambles the phase of the signal beam. The nonlinear interaction between the meter and signal beams, causing a correlation between the signal intensity and the meter phase, also correlates the signal phase and the meter intensity. In monitoring the meter phase we discard information about the meter intensity and hence introduce additional uncertainty into the signal phase. At the quantum level this is simply a manifestation of complementarity, in that to measure one observable, the amplitude, we induce an uncertainty in the complementary observable, the phase.

In an interesting variation on the QND theme, Grangier and colleagues also used a nonlinear optical crystal to produce two amplified copies of the information contained in one quadrature component of an input signal (J. A. Levenson *et al. Phys. Rev. Lett.* **70**, 267–270; 1993). Thus an amplitude-modulated signal is converted into two beams, each more intense than the original and carrying the same amplitude modulation. Observation of the amplitude of one beam reveals the modulation carried by its partner. This is not strictly a QND measurement because the signal after measurement does not have the same amplitude as it had previously. But the fact that it is more intense might make this technique useful in overcoming losses.

Optical QND measurements allow us to approach the ideal quantum measurement, although there is still some degradation of the signal — the quantum optical tap drips. Given time, advances in the development of nonlinear optical materials should provide the necessary washers. □

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50,000-year-old Americans of Pedra Furada

Paul G. Bahn

LONG-awaited data from the Brazilian rockshelter of Pedra Furada have at last been made public, and they constitute convincing evidence that human occupation of the New World extends back at least 50,000 years. In defending his doctoral thesis¹, Fabio Parenti, co-excavator of Pedra Furada with Niède Guidon, publically presented his analysis of the site and its contents to a jury of examin-

being followed by four of analysis and laboratory work. No site had yet met all criteria necessary to convince sceptics that humans had been present in the New World so far back in the Pleistocene⁴, so his task was clear from the outset. It was made all the harder because the sediments of the sandstone shelters of the Piauí region have destroyed all organic materials (other than



The rockshelter at Pedra Furada viewed from the gate.

ers in Paris on 15 February. His defence was successful. The upshot will be the opening of a new era of investigation into the 'first Americans'.

The sandstone rockshelter of Pedra Furada is one of several hundred painted shelters discovered and studied by Guidon in the Piauí region of north-eastern Brazil. In 1978 she began excavations in the site in order to date its rock art, which was confidently assumed to be of Holocene age (that is, less than 10,000 years old). When radiocarbon dates of Pleistocene age, extending back more than 30,000 years, started to emerge from the stratigraphy², the site and its excavator were thrust into the forefront of the debate in which one side (primarily North American) insisted that there was no human occupation in the New World before 12,000 or at best 15,000 years ago, and the other accepted far earlier dates from Pedra Furada, or sites at Monte Verde in Chile³ and elsewhere.

Parenti commenced work at Pedra Furada in 1984, four years of digging

charcoal fragments) in pre-Holocene levels. In addition, the Pleistocene levels of Pedra Furada contain tools made only of the quartz and quartzite pebbles from a conglomerate layer above the sandstone cliff, and pebble tools are notoriously difficult to differentiate from naturally broken stones.

Parenti's primary aim, therefore, after erosional, geomorphological and sedimentary study of the site and its surroundings, was to distinguish between human and natural agencies in terms of the site's contents in general, and of its lithic objects in particular. The stratigraphy comprised mostly sand as well as sandstone plaques that had fallen from the walls, with occasional rubble layers. It was a natural rubble 'wall' in front of the shelter which preserved the sediments within: in Piauí, only sites with such walls or those in protected locations contain Pleistocene layers. In all other shelters, the Pleistocene layers have been removed by flooding, because in Piauí that period was far more humid than the Holocene.

Within the Pedra Furada shelter, scattered through the stratigraphy, Parenti has identified over 150 'structures' — that is, arrangements of sandstone plaques, of pebbles, or of both. His analysis indicates that they cannot be natural, as they have no correlation with the areas where they would have fallen. The stones have been selected and arranged, though some pebbles subsequently moved since they can easily roll (the site has a 10° slope from east to west). It is impossible for the pebbles to occur naturally within the shelter; they would have fallen down the two watercourses in the cliff-face and along the shelter's drip-line outside the rubble wall⁵; besides, some circular arrangements of pebbles occur immediately adjacent to the shelter's back wall, some 15 metres from and upslope of the drip-line.

Parenti's basic rules for recognizing human agency in these structures are probably valid, but even when he applied the most stringent criteria he was left with no less than 50 Pleistocene structures which would be attributed to human agency on archaeological sites anywhere in the world; some contain traces of heating, such as burned stones and fragments of charcoal, and have been interpreted as hearths.

Where the pebbles are concerned, Parenti conducted a study of 3,500 stones fallen from the clifftop, and found that when they break — which is rare — the natural flaking never affects more than one side, never removes more than three flakes, and never produces 'retouch' or 'micro-retouch'. These observations became his benchmark for recognizing human artefacts at the site. Of some 6,000 pieces definitely considered to be tools, no less than 900 came from the Pleistocene layers (quartz and quartzite continued to be worked and used in the same way in the Holocene, but easily identifiable chalcedony pieces account for the high number of definite tools in that period). Even if the most stringent criteria of human agency are applied to the collection, Parenti has 595 Pleistocene pieces that he considers definite. Thousands more pebbles are ambiguous, and could be either natural or man-made.

Finally, the site also has a coherent series of 54 radiocarbon dates from 5,000 to 50,000 years before present. Thermoluminescence dates will be forthcoming.

The thesis-defence lasted for four hours (the thesis itself is a four-volume monster, weighing 7 kg). The combined experience of the jury is a measure of the quality of the interrogation. The panel was presided over by Yves Coppens, the palaeoanthropologist, and consisted of Niède Guidon herself; Claude Guérin, the palaeontologist in charge of studying the faunal remains from Piauí's

limestone sites; Jean-Philippe Rigaud, an expert on the Palaeolithic rockshelters and stone-tool industries of south-west France; Jean Chavaillon, an Africanist and expert on archaic stone tools; and Danièle Lavallée, a specialist in Andean prehistory. All six were convinced by Parenti's data, even though some (notably Lavallée) admitted to having had strong doubts about Pedra Furada in the past, and some were familiar with — and equally unconvinced by — the material from other controversial New World sites such as Calico, Pendejo and Old Crow. The thesis has effectively been through stringent — and public — peer review.

Regardless of the lack of early sites in North America, there is now solid archaeological evidence for a human presence in the New World tens of thousands of years ago. All other issues — such as when or how many times the Beringia land-bridge may have been

crossed, or the technological origins of the Clovis point, or why the Piauí stone tool industry is so archaic — become secondary to that. There will no doubt remain sceptics, especially in North America. But in December an international meeting is to take place in Piauí to which the foremost of them have been invited. Seeing may then be believing. □

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PSYCHOLOGY

Weighing of the talents

John Sloboda

WHAT determines whether people make exceptional contributions to a particular field of endeavour? High scholastic grades or psychometric measures of intellectual ability — such as IQ test scores, or other estimates of general cognitive activity ('g') — are by no means the best predictors of eventual excellence. Talent comes in many forms, and such qualities as perseverance, curiosity and self-confidence, as well as relevant experience, are often more crucial to high achievement.

Such was the seemingly uncontentious message to emerge from a recent meeting* (though very little else in this field can be described as uncontentious). The underlying question to be addressed was an age-old one, that of whether individual differences in achievement are best accounted for in terms of genetic differences or environmental differences. A bald 'either-or' stance is inappropriate — there is general acknowledgement that the characteristics of an individual are a result of an interaction of genetics and environment. There are well-established methods for estimating the relative contribution of heredity and environment to a behavioural measure within a population, by examining the degree to which it varies among people of greater and lesser degrees of genetic and environmental relatedness (for example identical twins, fraternal twins and children raised apart from their

biological parents). These studies have typically shown contributions of heredity to IQ of the order of 50 per cent¹.

There is an unhappy tendency to jump from such findings to the conclusion that genius is in some sense predetermined in one's genes, and that, no matter how favourable the environment, a person without a special genetic 'spark' will never reach the heights of achievement. There are several cogent objections to such a sweeping conclusion, amounting, in the words of one participant (H. Gardner, Harvard University), to a denial of the "hegemony of g".

In the first place, to show that some behavioural measure is heritable does not imply that it is immutable. Several speakers referred to *mono-savants*, individuals of low IQ who nonetheless show outstanding performance in one specific area of expertise². There are, for example, *savants* whose abilities to memorize music equal or even outstrip the feats reported of Mozart. It is clear that these levels of performance were achieved through continual practice, even if, as some at the meeting claimed, individuals with high IQ might have achieved the same levels of performance with less effort.

Second, familial studies provide only a crude gross measure of similarity of genetic material between different individuals, making detailed causal explanations of correlations between genetics and behavioural outcomes impossible. However, advances in molecular gen-

RÉSUMÉ

Bright spark

If you can make lasers with photons, why not with pions, subatomic particles created in high-energy collisions? Like photons, pions have integral spin, which means they obey the laws of Bose–Einstein statistical physics. One consequence of these rules is that the emission of one boson particle encourages the emission of another — that is how a laser amplifies light radiation. The trouble is calculating what actually happens in the strongly interacting nuclear plasmas generated in high-energy collisions. But S. Pratt has succeeded in devising a fast program to do this (*Phys. Lett. B* **301**, 159–164; 1993). Collisions using relativistic lead nuclei, planned for CERN in 1994 to recreate the conditions of the Big Bang, should also create the circumstances necessary for pion lasers, he estimates.

Protection business

OUR DNA is vulnerable to many unwholesome influences, and were there no mechanisms for scanning the genome for lesions and then repairing them we should soon fall prey to such conditions as xeroderma pigmentosum, which often leads to skin cancer. Chu and his associates identified a protein missing in one variant of the disease and have now taken the first steps towards characterizing its action (B. J. Hwang & G. Chu *Biochemistry* **32**, 1657–1666; 1993). It is a monomer, found only in the nucleus, and merely two molecules per megabase are needed to maintain constant surveillance of the DNA. This is because it binds to damaged nucleotides more strongly than to pristine ones by six orders of magnitude. The supposition is that having found its target it attracts other proteins that effect the process of excision and repair.

No two ways

MULTIFACETED buckminsterfullerene is now finding its way into electronic devices. A. J. Heeger and colleagues have made a diode by sandwiching a layer of C₆₀ and a layer of the conjugated polymer MEH-PPV between gold and indium/tin oxide electrodes (N. S. Sariciftci *et al. Appl. Phys. Lett.* **62**, 585–587; 1993). The polymer is a semiconducting material, already used in prototype devices (Heeger's group has made a flexible light-emitting diode with the material). And C₆₀ has been shown to be an 'n-type' semiconductor, able to accept electrons from p-type materials (such as MEH-PPV). Having seen photoinduced electron transfer at MEH-PPV/C₆₀ interfaces, Heeger and colleagues set about making the *pn* diode. As hoped, they found the forward current to be 10⁴ times the reverse current, and illuminated, the diode gives photocurrents and photovoltages.

* Ciba Foundation Symposium No. 178, *Origins and Development of High Ability*, London, 25–27 January 1993.

etics make possible studies that hold out the prospect of discovering whether there are any specific genes that contribute to differences in IQ (R. Plomin, Pennsylvania State University). It is almost certain, however, that no single gene contributes more than a tiny portion towards the overall variability of IQ. This means that traditional methods of linking genes to phenotypes must be modified. Plomin's group employs a technique called allelic association, which uses large samples of unrelated individuals at the two extremes of the IQ range, and analyses DNA markers in or close to genes having products of *prima facie* neurological relevance (such as dopamine receptor protein and neurofilament protein). The first results using this technique are expected to be published during the coming year.

Third, IQ tests were specifically designed to help predict scholastic achievement, which usually requires specific analytical skills, measured through pen-and-paper tests. Achievement in many endeavours requires other attributes, such as creativity or practical problem-solving. Many of the scholastic tests used to determine entry to higher training have little or no long-term predictive value; for instance, the Graduate Record Exam (GRE), used widely in the United States to select students for graduate study, fails to predict any important outcome other than first-year grades (R. Sternberg, Yale University).

A study of 34 successful mechanical inventors (N. Colangelo, University of Iowa) reinforces this point. None of them excelled at school, won any academic honours, or occupied any positions of leadership. Instead, they all grew up with easy access to materials and tools (many were raised on farms). They began tinkering with things at an early age, and were as likely to take toys apart as to play with them. Their invention was often sparked by the desire to make a household task less of a chore, and by the time of the study the average number of patents filed was 20. Several were millionaires.

Fourth, there is converging evidence that specific biographical and environmental factors are necessary for the development of high ability, such as arduous practice (K. A. Ericsson, Florida State University), motivation for the subject area and family support (M. Csikszentmihalyi, University of Chicago). Biographical studies of large numbers of similar individuals, supplemented by day-to-day tracking through diaries or event sampling, are yielding rich and specific environmental hypotheses which cannot currently be matched by genetic explanations of equal sophistication. For instance, there are simple but effective ways in which parents can secure sub-

stantial and long-lasting gains in the speech of their children through a few minutes each day spent in structured, interactive language games (W. Fowler, Center for Early Learning and Child Care, Cambridge, Massachusetts)³.

The symposium revealed many unresolved scientific challenges. I shall mention two. Different types of high ability require different personal characteristics and 'enabling' conditions, both within certain pursuits and between them. For instance, the individual who redefines a field (the 'genius') may be produced by a very different route to that taken by a successful practitioner who is content to work within the existing constraints of the same field (the 'expert'). What those routes are remains unclear. The same applies to the manifestly different resources and processes required for the creation of a permanent work, such as a symphony or a painting, compared with those involved in bringing off a 'high stakes' performance, such as a successful military campaign (H. Gardner).

The second challenge comes in a variety of forms, and is the need for adequate controls in these studies. Several of the research projects reported at the symposium showed what high achievers had in common. What they did not demonstrate was that appropriately matched low achievers lacked these common features. Although there is a whole range of methodological, conceptual, and practical problems in providing adequate controls, it is clear that some avenues of progress will remain blocked until they become available.

Despite intellectual acknowledgements of the essential duality of the origins of high ability, most individual researchers are emotionally — sometimes passionately — attached to the defence of one extreme. Maybe we should welcome such polarization, for vigorous defence of extreme and sometimes unpopular positions may well be a component of the motivation that spurs creative thinkers forward. But passion within a scientific community is one thing, outside it is another. In the wider world people have done massive violence to one another in the name of such extreme beliefs, and those of us who choose to work in this potentially explosive area bear the responsibility of ensuring that our work does not fan the flames of intolerance. □

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Slipping along

As a snail or a slug drags itself over the ground, it leaves a trail of slime behind it. This subtle lubricant must save the creature more energy than it needs to make and discard it along its path. Daedalus is now applying the same strategy to those other victims of excessive skin-friction, ships and aircraft.

What sort of slime best lubricates a metal surface? Daedalus recalls the metalocenes — compounds in which a ring of carbon atoms is jointly bound to a metal atom. The ring is loosely coupled to the metal: it can spin freely, for example. Daedalus reasons that such a carbon-ring molecule should be able to wander about a metal surface, stepping sideways from atom to atom. A molecule with several carbon rings, their spacing poorly matched to the atoms of the surface, should 'skate' over it quite freely. As it moved, some rings would bind while others disengaged; so its energy of binding would be almost constant for all positions.

A metal surface, coated with such a bound but perfectly mobile monolayer and placed in an airstream, should lose all viscous drag. Viscosity arises from the sideways diffusion of molecules between adjacent streamlines of different velocity; but the monolayer cannot diffuse away from the surface. It will simply be swept over the metal surface at the speed of the airstream. By travelling freely with the moving air, it will abolish drag.

A dragless, monolayer-lubricated aircraft will be wonderfully economical. It will need to be fitted with special ports along all leading edges, to replenish the lubricant monolayer as fast as it is swept downstream over the metal skin. Along its trailing edges, local heating elements will boil the lubricant away as fast as it arrives. A monolayer contains so few molecules that even a transatlantic flight would disperse a mere few litres of lubricant: comparable with the oil lost by the engines during the flight. The same system on a smaller scale could lubricate the fan and compressor blades of those engines. Enormous savings in fuel would result. The bare, polished metal skin of the craft might offend some airline operators, who would have to abandon the tasteless colour schemes in which they now paint their fleets — at least until a monolayer-lubricated paint can be invented.

The trick should also work with ships. Exuded continuously from the bow, the monolayer will sweep freely back towards the stern, abolishing skin-friction. Barnacles and limpets will also slide along with the monolayer and find themselves tumbling helplessly in the vessel's wake.

David Jones

Heisenberg and the bomb

SIR — Walker¹ misunderstands some essential issues involved in the Farm Hall documents, asserting that Werner Heisenberg's "competence" was somehow at issue in an extended exchange with Samuel Goudsmit, the physicist who led the Allies' *Alsos* mission into Germany. Goudsmit, who did not equate genius with infallibility, set Heisenberg in the company of Einstein and Bohr², but questioned whether Heisenberg and his wartime colleagues had reached a working understanding of fast fission weapons. A claim to such knowledge was implicit in the suggestion that some of the German physicists had refrained from building bombs for the Nazi regime on moral grounds³.

The *Alsos* mission discovered evidence of considerable confusion in both the administration of the several competing German uranium projects and in their physics, including a secret report by Gerlach from late 1944 alluding to nuclear explosives involving tons of uranium, rather than the tens of kilograms fast fission weapons actually require. Objecting that Goudsmit's book *Alsos*⁴ had overstated the case, Heisenberg insisted that he, at least, and some other theoreticians in his circle had understood the key issues of fast-fission physics and plutonium breeding. Like any practicing scientist, Goudsmit distinguished between superficial speculation and the kind of working knowledge from which practical consequences can flow, and carefully analysed the record for evidence that the Germans possessed such knowledge. Goudsmit did from 1949 onwards⁵ acknowledge Heisenberg's awareness of plutonium breeding as a theoretical possibility (the Germans did not succeed in creating even microscopic quantities of the element with which to experiment). No evidence that Heisenberg brought forward, however, documented the clear conception of a fast-fission bomb.

Heisenberg's conversations at Farm Hall reveal now, as they did to Goudsmit when he reviewed them in 1945, the thinking of a physicist who has considered the broad possibilities of nuclear weapons and some of the difficulties involved in their construction, but whose picture of how they would work is not clear. This becomes especially evident in Heisenberg's response at Farm Hall on 6 August to the news of the Hiroshima explosion. When he moved from general comments to actually estimating the minimum size of a uranium bomb, that day, his reasoning revealed some basic misconceptions about fast-fission bomb physics. Heisenberg's model was a reaction spreading from the centre to the

periphery of a uranium-235 sphere, fissioning 10^{24} uranium-235 nuclei in the process. He determined the minimum size of the sphere by requiring that diffusing neutrons must collide with and fission this many nuclei before reaching the surface. On that basis, he estimated the radius of the sphere as 54 cm, which he said gave a minimum mass of 1 ton (Actually, this much uranium weighs about 13 tons. Walker, inexplicably, reports Heisenberg's estimate as "hundreds of tons"). The appropriate criterion for a self-sustaining reaction, however, is that neutron generation exceed neutron loss through the surface, which gives a very different result. Nor do actual fast-fission explosions go to completion: outward expansion of the fissioning material halts the chain reaction very quickly — a decisive concern in fast-fission bomb design, which Heisenberg assures Hahn applies only to slow-neutron chain reactions (The Hiroshima bomb, in fact, fissioned only about 2% of its uranium⁶).

The disparity between the actual (bare) critical mass of 60 kg and Heisenberg's working value of a ton or more helps, of course, to explain his utter incredulity at the news of Hiroshima, because it defines the difference between a dauntingly difficult task and an impossible one. (The Allied project, ironically, gained decisive impetus when Frisch and Peierls in early 1940 underestimated the critical mass as 600 grams and so made the goal seem less difficult⁶.) Heisenberg, a week after the jolting lesson in critical mass provided by Hiroshima, revised his first, ill-conceived estimate, and presented a more elaborate calculation, which placed the critical mass at tens of kilograms. His lecture at Farm Hall on 14 August, however, is a primitive, unsophisticated treatment of the subject, treating a bomb as though it were a reactor. A second week's thought would have shown Heisenberg the unreliability of his argument and allowed him to discover some mistakes (using the total cross section to determine the mean free path introduces a spurious factor of one half in the critical mass). Walker, however, counts the lecture as proving Heisenberg's mastery of fission bomb physics, as if being able to arrive at a rough understanding after being given the fact of the bomb were the same as possessing it beforehand.

In Walker's view, the different courses of the German and Allied programmes can be fully explained by a single, well-informed decision taken by German Army Ordnance on the basis of wartime priorities not to proceed with a full-scale effort. Beyond oversimplifying things,

this reasoning imputes to the Germans an understanding of the implications and difficulties of a commitment to build bombs that is obvious now but was not then. The result is a view that excludes from examination the large and consequential differences between the situation of the Allied scientists and that of their counterparts under totalitarianism, without which this history becomes merely an exercise in charting the German bureaucracy and of guessing what the German physicists might have done had they known more and been given better support. These are just the kinds of errors and confusions we hope historians will protect us *against*.

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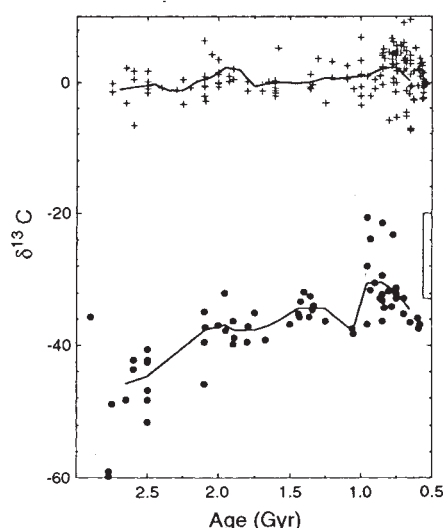
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Proterozoic carbon cycle

SIR — Des Marais *et al.*¹ argue persuasively for a non-uniformitarian view of the Proterozoic carbon cycle, envisioning a substantial buildup of the sedimentary organic carbon reservoir between 2.5 and 0.6 Gyr (billion years) ago. Having in its favour several elements of plausibility, the new hypothesis should stimulate the field; I propose that some residual questions be viewed in this light.

An uncomfortable corollary of the new concept is the relative constancy of δ_{carb} (the natural abundance of ^{13}C in marine sedimentary carbonates) in the face of a pronounced increase of ^{13}C abundance in organic matter (δ_{org}) over the same time interval (see figure). Defining δ_{in} as the ^{13}C abundance in the total carbon input to the atmosphere-ocean system and f_{carb} and f_{org} , respectively, as the fractions of carbon buried in inorganic and organic form ($f_{\text{carb}} = 1 - f_{\text{org}}$), then keeping δ_{carb} relatively stable within the constraints of the crustal carbon isotope mass balance, $\delta_{\text{in}} = f_{\text{carb}}\delta_{\text{carb}} + f_{\text{org}}\delta_{\text{org}}$ would require that increasingly larger fractions of isotopically heavier C_{org} go along with increasingly smaller proportions of C_{carb} with quasi-constant δ_{carb} (or, equivalently, the



Carbon isotope ($^{13}\text{C}/^{12}\text{C}$) composition of sedimentary carbonate (δ_{carb} , crosses) and of organic carbon corrected for postdepositional alteration (δ_{org} , circles) over Proterozoic time¹. Note the relative constancy of the isotope age function for carbonate and pronounced time trend for organic carbon.

product $f_{\text{org}}\delta_{\text{org}}$ in the equation must have been constant through time).

Although decreasing isotope fractionations between C_{org} and C_{carb} could be accounted for by an assumed decrease of atmospheric CO_2 pressures, any causative link between the magnitude of biological fractionation and the quantity of carbon stored as sedimentary organic matter (so as to keep $f_{\text{org}}\delta_{\text{org}}$ constant) seems to involve a fair amount of teleology. In any case, a delicately tuned inverse relationship between biologically mediated carbon isotope fractionations and the size of the sedimentary C_{org} reservoir with the result of a near-stabilization of a single selected parameter of the system (δ_{carb}) remains shrouded in mystery.

Although a theoretically plausible (but not proven) decrease in atmospheric p_{CO_2} over the time interval in question might have provided the CO_2 concentration effect supposedly responsible for the δ_{org} increase of the revised age function, a large part, notably of the older values, clearly surpasses the average intrinsic maximum fractionations of some -30‰ of ribulose biphosphate carboxylase² as the main carboxylating agent, suggesting instead an involvement of methylotrophic pathways.

For a final assessment of the proposed age trend in biological isotope fractionations, the buck probably has to be passed to enzymologists, who might be able to judge the feasibility of time-directed evolutionary changes in the isotope-discriminating properties of carboxylating enzymes. Further attention to these points, and perhaps other selected background parameters (including the

axiom that life, in its reckless striving for self-expression, always tends to sequester the maximum possible quantity of carbon permitted by nutrient availability), should help to test a possible conversion of the new concept from a captivating working hypothesis to an internally consistent cycling model.

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DES MARAIS ET AL. REPLY — We summarized the evidence that δ_{org} increased and that net isotope discrimination ($\delta_{\text{carb}} - \delta_{\text{org}}$) decreased substantially during the Proterozoic¹ (see figure left). These trends, which emerge from δ_{carb} and δ_{org} data obtained by several laboratories from hundreds of stratigraphic units, provide direct evidence that the carbon cycle changed. If δ_{carb} was relatively constant, as Schidlowski notes, then isotope mass balance requires that the fraction of carbon buried globally as organic matter, f_{org} , increased. Rather than interpreting the relative constancy of δ_{carb} or the decline in net discrimination, we explored instead the magnitude and episodicity of this increase in burial of organic matter, and its implications for the atmosphere and biosphere. We can, however, point out that no available evidence requires that the carbon cycle was unchanged during the 2 Gyr spanned by the Proterozoic. In fact, the trends we identified fit well with biological and environmental developments for which independent evidence exists.

How can the isotope trends for organic matter and carbonates be so different? The mass balance equation illustrates that δ_{org} and δ_{carb} respond differently to simultaneous changes in isotope discrimination and relative rates of organic matter burial. Specifically, decreasing discrimination and increasing organic burial both cause δ_{org} to increase, but they exert opposing effects on δ_{carb} . δ_{carb} can be evaluated over the interval 2.5 to 0.8 Gyr ago, using the trend in declining discrimination presented in our article¹, and estimating the increase in organic burial rate using the principle³ that changes in the crustal organic matter reservoir are stoichiometrically coupled to changes in the oxidized reservoirs, and then assuming that the reservoirs of free oxygen and sulphate increased largely during the Proterozoic (see ref. 1). The net effect on δ_{carb} between 2.5 and 0.8 Gyr was less than 1‰ . This small, long-term trend is considerably less than the scatter of δ_{carb} values observed, therefore it is hard to detect. The δ_{carb} trend was insensitive to a significant long-term change in the

carbon cycle!

Actually, δ_{carb} has not been perfectly constant over time (see figure). The variations indicate that the isotope consequences of changing biogenic isotope discrimination and rates of organic burial often were not precisely balanced over timescales shorter than a few hundred million years. Mechanisms producing a tight linkage between isotope fractionation and organic burial are thus not required.

At least two mechanisms can be envisioned which plausibly linked isotope fractionation and organic burial over timescales longer than 100 Myr. First, in a low- O_2 , late Archaean to early Proterozoic world, organic matter, already depleted in ^{13}C because of discrimination during photosynthetic carbon fixation, became even more depleted by globally significant recycling of ^{13}C -depleted biogenic methane⁴. Increased rates of organic matter burial caused an increase in environmental concentrations of O_2 , sulphate and ferric iron. This in turn limited net discrimination because it suppressed methane recycling. Thus, changes in net discrimination might constitute additional evidence for oxidation of the environment. Second, increased rates of continental erosion augmented both rates of organic burial and rates of CO_2 removal from the atmosphere. Declining atmospheric p_{CO_2} would also have decreased isotope fractionation during photosynthetic carbon fixation⁵⁻⁷.

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Lipid diffusion in neurons

SIR — Kobayashi *et al.*¹ suggest that the compositional differences between the axonal and somatodendritic domains of neuronal membranes are maintained by a diffusion barrier at the axon hillock. This novel suggestion is based on observations of the movement of fluorescently labelled lipids inserted exclusively into the axons of neurons infected by viruses. Although the fluorescent lipid is mobile in the axonal membrane, labelling is abruptly interrupted at the point where the axon emerges from the cell body; the cell body and dendrites do not appear to become labelled even after an hour of observation.

Using analysis similar to that developed for quantifying diffusion from a spherical cell surface to a tubular projection², we have calculated the amount of lipid that would diffuse from the axon to cell body after 1 h in the absence of a barrier, for lateral diffusion coefficients ranging from 10^{-9} to 10^{-8} cm² s⁻¹ (see figure). For a lipid diffusion coefficient of 6×10^{-9} cm² s⁻¹, as measured in spinal cord neurons³, only 9% of the lipid initially present in an axon of 100- μ m length would diffuse into the cell body after 1 h. Under these conditions, labelling in the axonal initial segment would not disappear, as was observed¹. Direct measurement of the diffusion coefficient of the fluorescent

lipids in the virus-infected neurons would permit evaluation of the contribution of a diffusion barrier in restricting movement of axonal lipids into the cell body.

We propose that a physical barrier may not be necessary to maintain the polarized distribution of cell surface molecules in axons. Mature axons in the central nervous system are often several millimetres long and can be as long as a metre in the peripheral nervous system. Assuming typical diffusion coefficients of approximately 10^{-8} cm² s⁻¹ for lipids and 10^{-9} cm² s⁻¹ for proteins (although many proteins move more slowly), and using the equation $t = L^2/2D$ (where D is the lateral diffusion coefficient, L is length and t the time taken for the molecule to move L), a lipid would take approximately 6 days to move 1 mm from the distal to proximal end of an axon, and a protein 60 days. Even for a shorter axon of 100 μ m, a lipid would take 80 min and a protein 14 h to diffuse this distance. It is generally assumed, at least in growing axons, that membranous material is added at the distal end of an axon. As approximately 50% of the membrane surface is internalized every hour (as measured in baby hamster kidney cells⁴, although a direct measure-

ment of the rate of membrane internalization has not been performed in neurons), the time a molecule spends in the axonal membrane before internalization will be much shorter than the time taken to diffuse long distances. Thus, the polarized distribution of a cell surface molecule could be obtained by targeting and inserting intracellular vesicles containing axonal molecules to a location in the axon that would prevent diffusion back to the cell body within a reasonable time. The significance of a physical barrier to lipid diffusion should be considered in the light of these geometrical considerations.

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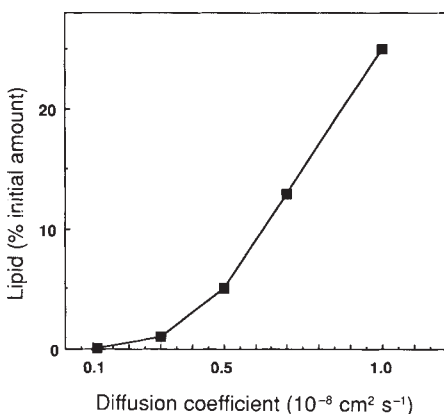
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Amount of lipid in the cell body after 1 h (as a percentage of the initial amount in the axon) against diffusion coefficient. The diameters of the cell body and axon were taken as 10 and 1 μ m, respectively, and the axon length as 100 μ m. The amount of lipid in the cell body after 1 h does not vary significantly for longer axon lengths or larger cell body diameters (not shown). Note also that for low diffusion coefficients (10^{-9} cm² s⁻¹), lipid that diffuses from the axon to cell body is restricted to the area of the cell body proximal to the axon, but for higher diffusion coefficients (10^{-8} cm² s⁻¹), lipid is almost uniformly distributed over the whole cell body surface.

Odour detection in bees

SIR — Breed and Julian¹ argue that data they obtained from controlled agonistic interactions of worker honey-bees "... suggest a system of ordering of priority of cues by the bees. ..." in the context of nestmate discrimination. This interpretation builds on the "hierarchy of importance of cues" hypothesis proposed by Carlin and Hölldobler² to explain nestmate discrimination behaviour in carpenter ants. But Carlin and Hölldobler's data are also consistent with a concentration or, more correctly, proportion hypothesis in which the recognition signature of the queen has diminishing influence as the size of the colony increases^{3,4}.

Similarly, Breed and Julian's data are also consistent with a proportion hypothesis which predicts that as the proportion of a salient odorant component increases in a blend, the blend is perceived as being increasingly similar to this component (as has been demonstrated for the case of blends of two fatty acids and blends of two *n*-alkanes⁵ and provided that the total concentration of the blend does not reach the point where the perception of quality is lost through saturation of the olfactory system⁶). Because the molecular mass, volatility, and receptor affinity varies among odorants, without the right sort of controls we cannot be sure that an odorant at one

concentration dominates another at the same or a different concentration. Our understanding of salience of odorants as cues is complicated because salience may vary with the genotype and experience of individuals. Further, we could misinterpret our results if we do not identify the threshold concentrations below which the odorants are not detected⁷. This point is not considered by Breed and Julian who hypothesize that "... the presence or absence of each cue, rather than the relative concentrations of cues, seems to have the greatest importance [in mediating kin discrimination]."

To exclude the effect of relative concentration, Breed and Julian would have needed to vary the relative concentration of their odorants in binary mixtures of the two, ranging from the hexadecane component increasing from its detectability threshold concentration to methyl docosanoate decreasing to its detectability threshold concentration. A proportion hypothesis — assuming concentrations c_1 and c_2 provide equally salient stimuli of odorants 1 and 2, respectively — predicts that worker bees would increasingly favour introduced bees exposed to odorant 2 over those exposed to odorant 1, if for some increasing value of α ($0 < \alpha < 1$) the receiving bees themselves had been exposed to a

$(1-\alpha)c_1:(1+\alpha)c_2$ blend of the two odorants. A complete switch in preference, however, might occur for a relatively small value of α . This, together with a possible greater salience for honey-bee workers of hexadecane over methyl docosanoate, could explain Breed and Julian's data under a proportion hypothesis.

A priority cue hypothesis requires that the components of a blend are individually perceived. In worker honey-bees, however, olfactory receptors do not function under a "labelled-line" paradigm but rather respond to classes of compounds^{8,9} to generate "across-fibre" patterns in the antennal lobe of their brains^{4,10}. Thus it is unlikely, in general, that bees can identify individual components in a stable blend. This is not to say that they do not perceive a blend that has a dominant component (in terms of salience) as being similar to that component on its own, or that they cannot identify components in blends that vary across space or with time¹¹. On the other hand, interaction effects¹² may cause the quality of a blend to be perceived as different from, rather than intermediate to, its component odorants, in which case the quality of the component odorants may be completely masked.

In making the statement "... the choice of [kin discrimination] cue compounds may be driven by the prioritization system. . .", Breed and Julian cite a study¹³ which actually supports a proportion rather than a priority hypothesis. In that study, aggression increases in a graded though asymmetrical way as differences in the discrimination signature are increased from purely genetic differences to both genetic and environmental differences. Further, data in a companion to that study¹⁴ indicate that the level of an aggressive response among individuals in a species of ant, as in the honey-bee¹⁵, is modulated in a graded fashion by both individualistic and *Ges-*

talt components of the nestmate recognition signature. These ant and honey-bee data are contrary to a priority cue hypothesis, which predicts that either the individualistic or *Gestalt* component should dominate so that no graded response is observed.

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BREED REPLIES — Getz, of course, is quite wrong: his critique fails on both empirical and logical grounds. Empirically, he suggests that hexadecane might simply be overpowering methyl docosanoate at the concentrations used, and that had a series of ratios been tried we might have found a pair of concentrations giving the contrary result. There are three problems with this argument.

First, the two compounds are equally effective in changing the recognition status of individuals. If hexadecane were more overpowering than methyl docosanoate, we would expect it to have a stronger effect in the controls; it does not. Second, the effect of hexadecane remains the same over a broad range of concentrations; it operates as an off/on signal rather than a graded signal over an order of magnitude of concentration differences (M. D. B. and R. Bowden, unpublished observations). The experimentally imposed cues do not work in a vacuum but co-occur with the cues that the bees already possess: if ratios were important the ratios between existing and imposed cues would assert themselves. Third, I tested tetracosanoic acid (which is equivalent in this system to methyl docosanoate) and hexadecane in 1:3 and 3:1 ratios, and found that 27.5% ($n = 51$) of the bees receiving a 3:1 ratio of tetracosanoic acid to hexadecane were attacked by bees treated with a 1:3 ratio, while the attack rate was 18.2% ($n = 39$) in controls (in which bees all received a 1:3 ratio). This difference is not statistically significant ($\chi^2 = 1.86$, d.f. = 1). This result is consistent with the data presented in our original paper.

The logical problem with Getz's ratio model is that as a bee flies to and from floral resources its surface is heated due to both internal heat production (from muscular activity) and to solar radiation. The thorax of a flying bee is usually several degrees warmer than the ambient temperature. Compounds that are known to be active in honey-bee recognition vary considerably in their volatility. As a flying bee heats up, concentrations of individual compounds will change differentially. Thus, the ratios of a bee arriving back at the colony may differ substantially from the ratios of

departing bees. Getz's model cannot account for how bees accommodate these changes.

Getz favours the ratio model but has no empirical evidence in this behavioural context to support his argument. He attempts to undermine our position by suggesting an impossibly difficult experimental design, based on the idea that if one tries enough different ratios and enough concentrations, ultimately evidence for a ratio model will appear. I suggest that the data in our original paper, perhaps strengthened by the additional information mentioned here, establish the principle of cue prioritization firmly enough that it can only be dislodged by an empirical demonstration to the contrary.

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Inside information

SIR — Gonnet and Benner¹ claim that words obtained from the one-letter code for amino acids in protein sequences "are candidates for the most unusable pieces of information". On the contrary, such words in a sequence can give valuable information about the protein. I have come across two such cases without even searching. The end of the sequence of the insulin-responsive glucose transporter (IRGT, Glut 4) from rat, mouse and man reads ...END (...Glu Asn Asp)²⁻⁶. The second case, which may not be obvious to non-Swedes, is in the sequence of the pig lactate dehydrogenase: ...SVIN... (...Ser Val Ile Asn... at positions 302-305)⁷, which in Swedish means 'pig', and which should leave no doubts about the protein origin. However, the chicken lactate dehydrogenase H-chain is also labelled SVIN (ref. 8), which calls into question the reliability of these built-in pieces of information.

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Tales of Jay Amber

Walter Gratzer

Cranks, Quarks, and the Cosmos. By Jeremy Bernstein. *Basic Books*: 1993. Pp. 240. \$23.

THE old guru Arthur C. Clarke once told Jeremy Bernstein that nothing had ever happens to a writer, for even the most calamitous experiences are in time transmuted into the raw material of his craft. During his double life, as physicist and writer, Bernstein has laid down a cellarful of treasure. In this, his latest collection of reviews and essays, many of them drawn again from that incomparable source of good writing about science, *The New Yorker*, he dispenses it in generous measure and with unfailing sureness of style. There are splendid anecdotes, some old, though always with a new gloss, and many new; there is here the story of the memorable confrontation between Pauli and Bohr, circling around the lectern in their urgent desire to inform the audience, like two ponderous men o' war manoeuvring for the weather gauge; of an exhausted Schrödinger, seeking refuge in his bedroom from Bohr's tireless persistence, but pursued thither by the unassuageable Great Dane. But he begins with a reminiscence about his start in serious writing; he pays tribute to the second editor of *The New Yorker*, William Shawn, who, in his famously diffident way, gave Bernstein the only lesson he ever needed in prose style and also dissuaded him from taking a pseudonym (Jay Amber, it was to have been — in German, Bernstein).

Bernstein believes that to write about science one should stay in touch with one's roots, and he has honoured this principle by rejecting the option, clearly at all times open to him, to become merely a writer. He is proud of his calling as a professional physicist — one who also writes. In a deeply felt appreciation of Primo Levi, he implies that Levi was mistaken in cutting himself off at the age of 58 from his profession as an industrial chemist, which had given him a base in the real world, to make more time for writing. As for C. P. Snow, his science was a sham, exposed as such in his last woeful foray into the realm of fact, *The Physicists*.

Bernstein's heroes — Lee, Yang, Mach, Gamow, Oppenheimer and, above all, Einstein — glide through these pages, now and then advancing to centre stage to point a moral or adorn a tale. Here you will find the true history of the celebrated paper by Alpher, Bethe and Gamow; there would, it seems, have been a fourth author (Herman), had he not resisted Gamow's

demand that he change his name to Delter. In the discovery of the primordial radiation, for which Penzias and Wilson were rewarded with the Nobel prize, Bernstein has uncovered other elements of comedy. Gamow and his colleagues deduced the existence of such a radiation and predicted its temperature; Penzias and Wilson were unaware of these publications and of the pre-occupations of cosmologists generally, so did not know what to make of the tiresome microwave background. A chance telephone call from a friend, who

"[Bernstein] takes a notably austere view of biography. The circumstances of scientists' lives with no bearing on their science are, he thinks, at best boring, at worst an embarrassment and an intrusion."

had a friend, who had heard a seminar in Baltimore by Peebles, put them in touch with Dicke's group at MIT, of which Peebles was a member. Dicke too had come to the conclusion that a residual radiation should exist, and Peebles had rediscovered, not having seen the original, the basis of Gamow's calculation. As F. H. Westheimer has truly said, a month in the laboratory can often save an hour in the library. Dicke and his friends recognized the significance of the background radiation, but Penzias and Wilson were unreceptive: Wilson was a disciple of Fred Hoyle and so in any case disposed to reject the Big Bang theory.

And yet the real discoverer, as Bernstein tells it, was arguably the astronomer, McKellar, who about 20 years earlier had observed a rotational splitting in a spectral line from cyanogen. Herzberg, in his classic textbook on spectra of diatomic molecules, had adverted to the rotational temperature of the cosmic radiation that had to be invoked to explain this splitting. He was writing some two years after Gamow and his associates had explained the significance of the temperature, but Herzberg had not read their paper, nor did they read his book. Scientists, Bernstein concludes, even the best of them, are frail vessels.

Bernstein has strong views about the history of science and makes known his disapproval of those who (like Stephen Hawking, in his opinion) do not take the

trouble to read the work of their predecessors. He takes a notably austere view of biography: he upbraids Watson for debasing the DNA story with talk of Cambridge *au pair* girls; Luria for his account in his memoirs of his nervous breakdown; Walter Moore for his candour about Schrödinger's priapic appetites; and especially Andrew Hodges for occupying himself in his biography of Turing with his subject's homosexuality. The circumstances of scientists' lives with no bearing on their science are, he thinks, at best boring, at worst an embarrassment and an intrusion. It is not clear whether he would lay the same rather bleak strictures on the biographers of, say, Dickens, H. G. Wells or Laurence Olivier; if so he would bankrupt publishers and deprive the literate population of one of the few remaining unpunished pleasures in this unforgiving world. Bernstein does, all the same, ruminate on Schrödinger's craven capitulation to political pressures, for there was indeed a strange contrast between his comportment on the Italian front in one of the most harrowing campaigns of the First World War and the abject manner in which he abased himself before the Führer, and his epigones in the University of Graz: physical and moral courage are evidently the products of different genes.

And now who is this sprightly ghost from a vanished world? Why, Bernstein's Harvard friend and the idol of all who were young in the sixties, Tom Lehrer. In the time of his fame it was rumoured that he was a mathematician of genius. The truth is that he was indeed a prodigy of sorts, who graduated from Harvard at the age of 17, but then he took 11 years over a PhD which in the end he abandoned. It was not that he preferred his other career as an entertainer, but rather that he became persuaded that his mathematical talents were insufficient to make any impact, and he opted instead for teaching: "I am not a mathematician, I am a *teacher* of mathematics". It brought to mind the preface of a maths primer that, according to his biographer, Robert Kanigel, first ignited the divine fire in the young Ramanujan, then still a schoolboy in a small town in southern India. It was evidently a remarkable book and its author was one George Shoobridge Carr, who had read mathematics at Cambridge. Abler hands than his, he wrote, might have done it better, but then abler hands than his could also perhaps have been more usefully employed. Teachers, alas, seem never to have set a much higher valuation on their craft than has society at large and especially the academic profession.

This is pretty much what Bernstein also says about women in science. His

exemplar is the mathematician Sophie Kovalevsky: born into the White Russian minor aristocracy, she studied algebra in secret by night, while her governess slept. There was never any question of a university education, but she had the good luck to find a patron, the German mathematician Weierstrass, who set aside his innate misogyny in the face of her evident brilliance. During her short and tempestuous life she gave herself only intermittently to mathematics and was toying with a plan to turn novelist when she died of pneumonia at the age of 41, her last sibylline words, "too much happiness". Bernstein hazards that Kovalevsky lacked the intensity of purpose that alone will liberate genius. Was it, as Einstein (appearing once more at Bernstein's shoulder) asserted, a feminine trait? We dare not even contemplate it; and besides, here is Einstein's reply to the sister of his close friend, Michele Besso, who wanted to know why her brother had made no famous discoveries in mathematics. "*Aber*, Frau Bice, this is a very good sign. Michele is a humanist, a universal spirit, too interested in too many things to become a monomaniac. Only a monomaniac gets what we commonly refer to as results". The melancholy implication is caught by W. B. Yeats:

The intellect of man is forced to choose
Perfection of the life or of the work.

Bernstein's view, which will outrage nobody, is that women are predisposed by social custom and the educational system to turn away from occupations that demand too narrow a focus; and, as that old reactionary, Dr Johnson, put it, "a man is generally better pleased when he has a good dinner on the table than if his wife speaks Greek".

Cranks, Quarks, and the Cosmos arrives finally at the cranks in an essay-review of Stephen Jay Gould's fizzing polemic *The Mismeasure of Man*. Bernstein sees off (as does Gould) the perpetrators of the soft and dark science that threw up a nexus between race and intelligence, and helped, in democracies as well as dictatorships, to legitimize much social evil. In this and in his other ruminations — on why for instance universities must teach science to *all* their students, not only to scientists (is there anyone listening in our academic groves and grooves?) and on why he did not become either a dean or a journalist — Bernstein's is the civilized voice of science. This is a wise, enlightened and entertaining discourse, and everyone will be the better for lending it an ear. □

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Advice matters

Harvey Brooks

Working with Congress: A Practical Guide for Scientists and Engineers. By William G. Wells Jr. *American Association for the Advancement of Science Press: 1992. Pp. 153. \$12.95 (pbk).*

THIS admirable volume was commissioned by the American Association for the Advancement of Science and the Carnegie Commission on Science, Technology, and Government. The author, William G. Wells, a professor of management science at George Washington University, is uniquely well qualified for the assignment, having served as chief of staff of the Office of Science and Technology Policy in the executive branch and as a staff director for key science subcommittees of the US House of Representatives, as well as an Air Force officer and a manager in NASA's Apollo programme. He worked on the guide with an *ad hoc* advisory committee of seven members, all with experience as staff members for congressional committees or congressional support agencies.

In the first half of the book, Wells describes the US governmental system with emphasis on the organization, procedures and personnel of Congress. The other half of the guide consists of specific do's and don'ts for scientists dealing with individual members of Congress or testifying before congressional committees. There is also a very useful glossary of terms relating to congressional procedures and offices, and several appendices of addresses and telephone numbers for key congressional committees and support offices, Washington offices of professional societies and some other relevant organizations.

The descriptive chapters include an excellent discussion of recent changes in congressional membership, committee staff and procedures, with mention of such important issues as the effects of divided government, the growth of staff and workload, the increased professionalization of staff, and the function and staffing of the principal congressional support agencies, such as the Office of Technology Assessment, the General Accounting Office, the Congressional Budget Office and the Library of Congress and its congressional information support arm, the Congressional Research Service. The modes of operation of all these offices are clearly and realistically explained.

The second part of the book is down to earth and practical, and is 'must' reading not only for scientists and engineers expecting to deal with Congress, but for almost any technical person

whose professional work is affected by government funding, regulation or policy. The message of this section comes through loud and clear. If scientists want to be taken seriously by congressmen, they must show that they not only understand the way Congress works and the pressures acting on individual congressmen, but also that they respect politics and the political process — especially the necessity of compromise among competing interests and political agendas. Scientists are expected to relate their policy proposals to the political agendas and constituency interests that are of daily concern to congressmen. This part of the book draws heavily on responses to postal questionnaires sent to congressmen and their staff and on extensive personal interviews, and is sprinkled with highlighted inserts quoting specific individuals, which bring home the general points in the text. A typical example is that of Senator P. Domenici: "In general we trust information from scientists. But to keep that trust you must clearly state which of your points is opinion, theory, or widely accepted fact." Wells also points out that there is a general "perception in the world of politics that a sizeable number of scientists and engineers believe they are 'above it all' and feel that being involved with politicians is inconsistent with the ethos of the scientific and engineering professions."

If I have any quarrel, it is that the author fails to distinguish sufficiently between situations in which scientists are interacting with Congress as advocates or critics of particular scientific research programmes ('policy for science') and where they are providing information or understanding relating to broader public policy issues in which technical input is important but not the sole consideration ('science for policy'). A little too much of the advice seems to be put forward with an implicit assumption that the scientist interacting with Congress is appearing on behalf of a particular scientific programme that he or she thinks is important, rather than trying to help inform a public policy decision in which science is only one consideration. It is unfortunate that this orientation of the discussion tends to reinforce the impression of many politicians that scientists can be treated as 'just another interest group'. Although this may sometimes be true, it is misleading to think of this as the principal role of scientists in the political process, even if the distinction between the two roles is not always completely sharp. □

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Small problems

Philip Ball

Nanosystems: Molecular Machinery, Manufacturing, and Computation. By K. Eric Drexler. Wiley: 1992. Pp. 556. \$49.50, £32.95 (hbk); \$28.95, £19.50 (pbk).

Nanotechnology: Research and Perspectives. Edited by B. C. Crandall and James Lewis. MIT Press: 1992. Pp. 381. \$39.95, £35.95.

Engines of Creation (Anchor/Doubleday, 1986), K. Eric Drexler's prophetic vision of the future of nanotechnology, arguably did as much to harm as to help its cause. It painted an exciting picture of nanoscopic machines that replicate, build crystalline rocket engines, repair cells and extend lifespans. But it was also infuriating, because it failed to give even the beginning of an explanation as to how these marvels might be achieved with meaningfully accessible technologies. *Nanosystems* purports to be Drexler's answer. Here you will find out how to make gears from precisely 3,557 atoms, sleeve bearings from 2,808, robot arms from about four million.

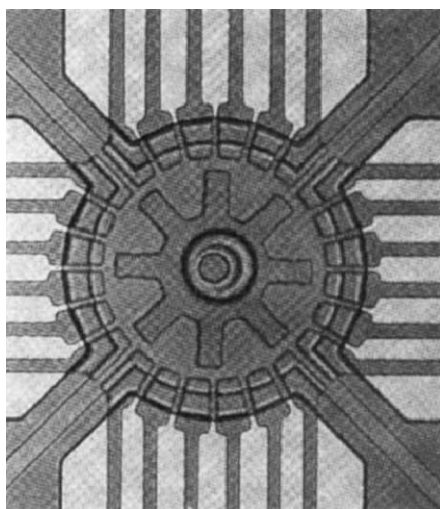
Or not. The schemes Drexler advances to make these nanocomponents are not even in the early stages of development. And no wonder: for they too involve nanomachines, which must be put together by other nanomachines. Breaking that circle is where the problems lie. For the most part, Drexler explicitly chooses to forget this difficulty and to assume that such things will become feasible, enabling him to reject the conditional and future tenses in favour of the present.

But Drexler is not unconscious of this leap of faith, and he includes several chapters aimed at discussing how the first steps might be taken using existing technologies, such as the atomic force microscope (AFM) and protein engineering. Yet because his vision of the nanoscale future is so limited, consisting solely of diamond-based ("diamondoid") machines performing every task by mechanical means, he places nanotechnology in a strait-jacket that prevents the ends and the current means from converging. His attempt to work backwards from operational nanosystems to realistic present objectives means that he overlooks the myriad other directions (many doubtless unrecognized as yet) that present-day molecular-scale engineering may take.

It also means that research that is of no obvious use in attaining his highly specific goals is neglected. Distressingly, this includes most of chemistry. Supra-

molecular and macrocyclic chemistry merit little more than a few paragraphs in the second appendix, and are then mentioned only to the extent of dismissing their "forward-chaining" approach (taking present synthetic capabilities as a point of departure) as ineffectual in the long term. So Drexler ends up proposing to do the hard way things that might be more readily (and more quickly) accomplished with a little chemical ingenuity.

Take Fraser Stoddart's 'molecular train', for example. Stoddart's elegant solution-phase synthesis gives a molecular 'bead' on a macrocycle, with docking points ('stations') where the bead is brought to rest by electrostatic interactions. Here, to judge from this book, is how Drexler might approach the



The real thing — a silicon micromotor.

problem. First you use an AFM-type cantilever arm to create a diamondoid carbon ring, by pushing the atoms together one by one by so far unknown "piezochemical" synthesis. Then you fashion a sleeve on the ring in a similar manner. Both structures are coated with fluorine atoms for lubrication. Docking of the train at a station is accomplished mechanically — perhaps with some kind of ratchet mechanism fashioned from a few hundred atoms. For Drexler, small is not so much beautiful as clumsy.

To be fair, this is because he sets his sights high. Drexler is after total control, unhampered by thermal disruption. This degree of control is, of course, what molecular biology achieves all the time, but Drexler seems to despair of our ability ever to learn from and to mimic nature, in favour of an approach whose feasibility has not even begun to be demonstrated. If, nevertheless, one accepts this as the premise on which the book is predicated, the challenge he sets chemistry is an intriguing one: can one build molecules by pushing atoms together? In effect, Drexler is proposing to invent a new chemistry, which he calls "machine-phase chemistry". But the

question is whether this challenge is worth taking up, rather than building on the achievements so far.

And these are largely overlooked in this book. The reader would emerge unaware, for example, that Don Eigler has already tried to push together a carbon monoxide molecule and an oxygen atom on a metal surface with the tip of a scanning tunnelling microscope (although they would not react to form CO₂). It is this sort of work that puts constraints on the possibilities, not interminable computer modelling. Microlithography is ignored, despite already being able to work at nanometre dimensions, and astonishingly we are told that the nanotechnological computer will be mechanical, like a Lilliputian version of Babbage's difference engine. There is next to no mention of fullerenes, perhaps the ideal nanoscale building blocks, and none of Sumio Iijima's carbon nanotubes, surely the closest we have yet come to nano-girders.

The most frustrating aspect of the book, however, is that Drexler constantly shies away from making a start, like an inventor endlessly refining his models for fear that the real thing will not fly. After presenting molecular modelling of his various cogs, gears and flywheels, he calls for more accurate simulation routines, not for preliminary experimental attempts at fabrication.

The narrow meaning of nanotechnology in Drexler's lexicon acquires more breadth in *Nanotechnology*, a book based on a conference that he organized in 1989. Supramolecular chemistry still doesn't get much of a look-in, but just about everything else does. Here, in a highly accessible format, is a discussion of atomic manipulation with the scanning probe microscopes, self-assembly in molecular crystals, protein engineering, micromachining and much else. It is to Drexler's credit that he assembled such an interdisciplinary collection of speakers, but sadly their contributions make it all the more obvious that Drexler's own approach almost uniquely lacks experimental results.

Will nanotechnology become an identifiable discipline? Rather, it may serve merely as an umbrella under which people with different aims and approaches will occasionally unite for a fertile exchange of ideas. Certainly it would be a shame if the term were to become shoe-horned into the restricted definition of the construction of tiny machines. So while Drexler begins the conference proceedings by warning us to be "harshly critical of anything that you hear called 'nanotechnology' — my own words included", he should beware too of painting it into too tight a corner. □

Philip Ball is an assistant editor of *Nature*.

All in the mind

Michael Morgan

Nature's Mind: The Biological Roots of Thinking, Emotions, Sexuality, Language, and Intelligence. By Michael Gazzaniga. *Basic Books*: 1992. Pp. 220. \$25.00.

MICHAEL Gazzaniga advances a 'selectionist' thesis of mental and neural development, as an alternative to the time-honoured philosophical dialectic between 'nativism' and 'environmentalism'. The key idea, which is by no means novel, is that environmental influences have no power to instruct the developing brain: they can only 'select' between alternative pathways of development that are specified by genetic factors, and ultimately by natural selection. As in the case of Edelman's 'neural darwinism', the selectionist model is strongly influenced by the clonal selection theory of the immune response.

Our understanding of the interactions between 'human nature' and the influences of child-rearing and education has always had implications that go far beyond the armchair and the laboratory. It is important that scientists and philosophers should comment on them in a responsible fashion, and a book surveying recent evidence is in principle to be welcomed. Unfortunately, this book seems to me to be confusing and unlikely to advance popular understanding of its topic to any great extent. It doesn't help that it is laced with political guesswork of the kind approved by the amateur psychologist Margaret Thatcher. Consider this shaft against social support systems, welfare and retirement homes:

An individual who cannot make an impact on his or her environment and exercise personal choice *will never feel the sense of control and self-sufficiency that has been demonstrated to be vital to physical and mental health*" (pp. 193, my italics).

How did we get from immunology and neuroscience to here? The answer is, unhappily, by a series of analogies and loose arguments that hinge upon a muddled use of the term 'selection'. In general style, the book belongs to the 'scientific heroes and villains' genre. The heroes are the immunologist Niels Jerne and "ten outstanding biologists, neuroscientists, and cognitive scientists" who met to discuss the issue in Venice. This happy band included *inter alia* "the smartest man in the world" (the late Leon Festinger); also Steven Pinker of the Massachusetts Institute of Technology "who is the world's expert on language and on just about everything else in cognitive science these days"; and

"this immensely talented man" Stephen Jay Gould, who enlivens the proceedings with a cathedral tour.

The villain of the piece is someone called "poor old John Locke", who was apparently so ill-advised as to believe that human minds are formed by their environment without the benefit of any preceding natural constitution. Presumably this poor old chap made his living by teaching Latin to stones and crustaceans. He would have had little in common with the empiricist philosopher John Locke (*An Essay Concerning Human Understanding*, 1690) who fully realized that the acquiring of 'ideas' depended on a pre-existing natural constitution. Discussing the secondary qualities such as colour, Locke said:

... it is no more impossible to conceive that God should annex such ideas to such motions, with which they have no similitude, than that he should annex the idea of pain to the motion of a piece of steel dividing the flesh, with which that idea hath no resemblance.

What Locke really didn't like was the view that the mind is implanted with suprasensory 'innate ideas', such as belief in the divine right of kings, which could be used to justify arbitrary authority. Against innate ideas he urged the cause of free thought and inquiry. It's a pity that Gazzaniga doesn't do justice to Locke's position, because to do so would take us to the heart of the real issue about nativism. To say that "the environment" can bring out in us only skills and ideas that we have the capacity to acquire is a truism: there is no evidence by which this version of the selectionist thesis could be refuted. It is a different, and much more serious, matter to say that all our adult cognitions are somehow already built into us at birth like a homunculus, waiting only for the environment to decide which ones will be expressed. Yet Gazzaniga sometimes seems to believe just that: "When we think that we are learning something, we are only discovering what has already been built into our brains" (p. 8). This really does sound like innate ideas, and poor old Locke might well have objected. However, when it comes to providing evidence for this striking claim, Gazzaniga is content to parade the standard findings that cognition and brain development are constrained by biological factors. The review of the evidence, for example of stereoscopic vision and of language development, are interesting and useful, but they fall far short of justifying the 'strong' version of selectionism with which the book begins.

Indeed, as the book progresses, the concept of selection gradually acquires mutations, so that it ends up encompassing any effect of previous natural selection on behaviour. Thus, there is a

genetic propensity to drug addiction, so "Selection theory would predict that the recidivism rate would be high" (p. 145). As a further example: "Daly & Wilson reveal a simple truth: Blood killings are extremely rare and, when they do occur, they have explanations in keeping with selection theory." Faced with such examples, I ended up being thoroughly confused about the meaning of selection theory. It is certainly not easy to relate to the clear example of immunology.

One might conclude that there is an urgent need for a book that examines the complex logical problem of nativism in the light of new findings from psychology and neuroscience. My suggestion is that such a book could not be satisfactorily written without attempting to come to grips quantitatively with information theory: how much of the information, in a mathematical sense, in a given cognition is provided by genetic programming, and how much by environmental input? If 'strong' selectionism is correct, this book may well be present in the brain of someone already, waiting only for the opportunity to be written. But what would be the point? If it is in your brain you know it already; if it's not, you can never acquire it. □

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Eco-efficiency

David R. Cope

Changing Course: A Global Business Perspective on Development and the Environment. By Stephan Schmidheiny with the Business Council for Sustainable Development. *MIT Press*: 1992. Pp. 374. \$34.95, £19.95 (hbk); \$16.95, £9.95 (pbk).

ALTHOUGH the imminent publication of the gigantic report of its proceedings may lead to a temporary revival of interest, the United Nations Conference on Environment and Development, held in Rio de Janeiro in June 1992, has disappeared as a subject of media attention more rapidly than anyone might have predicted. Preoccupied by concerns with economic recession and monetary instability, governments have looked hard at commitments made at the time of the conference. The UK government is examining closely its proposed "Darwin Initiative", while the Japanese government has shelved a comprehensive environment bill planned for the current parliamentary session.

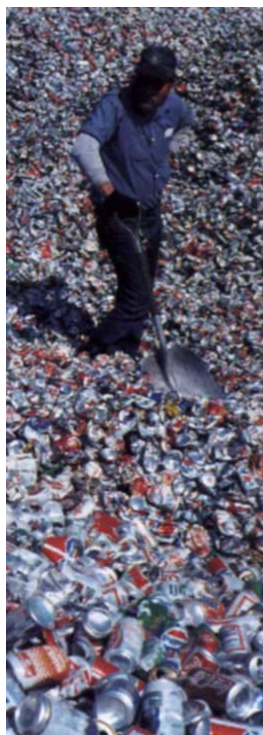
While governmental, and in particular

intergovernmental, action may have been the main intention of the conference, it also spawned initiatives by non-governmental agencies. Perhaps the mantle of responsibility for taking forward the aims of the conference will fall, at least temporarily, on their shoulders and, in particular, on the private enterprise sector, whose main input came from the Business Council for Sustainable Development (BCSD), through its report *Changing Course*. Indeed, the report virtually seizes the mantle. It argues that the control of change (the "changing course" of the title) by business is more efficient and cheaper than control by government. As for the role of the BCSD itself, it claims, in setting out to "present a global business perspective" on environmental issues, that it offers "more than a . . . book, a process" and promises continuing output, particularly in Africa, Asia and Latin America.

The BCSD was set up in 1990 to prepare input to the conference and was composed of some 50 leading business people, mainly from countries belonging to the Organisation for Economic Co-operation and Development (OECD), but also from non-OECD states such as India and Kenya. The council also intended to have Eastern European representatives but this proved to be "a challenge beyond us". Among the members, perhaps the best known are Bill Ruckelshaus, former director of the US Environmental Protection Agency, and Carl Hahn of Volkswagen. Alongside them served chairmen and chief executives from major international companies such as Dupont, Dow, Asea Brown Boveri, Nissan, Mitsubishi and Royal Dutch Shell.

The report falls into two parts. The first ten chapters examine a range of considerations germane to the global business perspective. These are followed by 38 case studies of individual companies, many of them represented on the BCSD. With such a large number of cases reported, it is inevitable that they are varied in detail, in the generalizations that they offer and, indeed, as examples of the full penetration of the sea change of thinking and practice that the first half of the book urges. The report acknowledges that they are not necessarily "models of perfection".

Although the case studies repay systematic examination, it is the overview in the first half of the report that makes this book distinctive. Chapters cover themes such as energy, renewable resource management, 'full cost pricing' (the inclusion of associated environmental damage in the price of goods and services) and the role of capital markets. The preface claims that a new perspective the BCSD has introduced is "tech-



To coincide with the Earth Summit, the United Nations Environment Programme together with Canon Inc held an international photographic competition on the theme "Focus on Your World". There were 32,000 entries from 144 countries. Shown here are *Can Bank Man* (A. Vohra, Canada) and *A Harmony — Beauty and Power* (N. Ghatak, India), two of the 214 outstanding entries exhibited at the summit and now collected together in *Your World* (HarperCollins, \$28, £14.95 (pbk)).

nology cooperation" between developed and developing economies. This covers a broader range of aims than technology transfer and emphasizes that the overall infrastructural and educational context in which a technology is deployed is as important as the hardware itself.

Actual technology transfers are best handled, the report maintains, by "business to business relationships". The report dismisses as "naive" the view that technology can systematically be moved on a concessional basis — concessions may be possible as a form of loss leader or to promote goodwill, but this must be decided by the enterprises with the proprietary rights to the technology concerned.

I searched with particular diligence for perspectives on environmental science and its relationship to the lead role claimed for business initiatives. If business is better able to handle uncertainties and more open to the challenges of change, then should it not be supporting the research necessary to clarify uncertainties and identify new challenges? Here the report is a disappointment. True, there are examples given of particular cooperative ventures, such as Mitsubishi Corporation's support of experimental reforestation using diverse indigenous species, but the most extensive discussion of environmental science is in a context that may raise a sense of anxiety among many practitioners.

This comes in a chapter on trade and the environment. The report (quite correctly) notes that environmental dimensions to international trade disputes are

increasingly likely (essentially because companies in countries with stringent environmental constraints will seek to protect themselves against competitors in countries alleged to have less onerous requirements). The BCSD calls for "international panels of scientific experts" to test the legitimacy of trade restrictions based on environmental grounds. Furthermore, it argues that "where environmental threats are particularly serious" the General Agreement on Tariffs and Trade "should adopt the precautionary principle, erring on the side of prudence". Prudence from one perspective is crying wolf from another. It is a brave scientist who claims to judge between the two. □

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Rio remembered

The 'Earth Summit' has already spawned several new publications. These include:

- *The Earth Summit* (Kluwer, £78, \$145), which contains the full text of the two international environmental conventions opened for signature, Agenda 21 and selected important ancillary documents;

- *The Rio Treaties of the Global NGO Movement* (Adamantine Press, 3 Henrietta Street, London WC2E 8LU, UK; £35), which brings together the text of all 29 treaties agreed by non-governmental organizations at the Global Forum; and

- *The Earth Summit: A Planetary Reckoning* (Global View Press, \$16.95 (pbk)), by A. Rogers, a personal account and analysis of the summit, including the UN documents, NGO treaties and speeches.

Colonial rule

Gene E. Robinson

Bees as Superorganisms: An Evolutionary Reality. By Robin F. A. Moritz and Edward E. Southwick. Springer: 1992. Pp. 411. DM195, \$129, £77.

THE superorganism is back, revived primarily by E. O. Wilson in the mid-1980s, after more than a 20-year absence, to highlight the importance of wedding reductionist analyses of individual insect colony members with a holistic appreciation of the colony. Recently others (D. S. Wilson and E. Sober; T. Seeley) have gone further, asserting that the superorganism is not only a useful metaphor, but a real biological entity. This is also Robin Moritz and Edward Southwick's thesis. One unintended lesson of their book, which focuses on honey bees, is that considerable problems emerge when attempting to move the superorganism from metaphor to reality.

One problem arises when interpretations of colony biology are forced to fit the biology of individual organisms. Moritz and Southwick sometimes resist this temptation, for example when describing how swarming in bees is not genetically equivalent to budding. But they succumb at other times, to erroneous or confusing effect. For example, they conclude that bee colonies sleep, on the basis of only W. Kaiser's suggestive evidence that individual worker bees show sleep-like states of inactivity. M. Lindauer's exhaustive observations, however, have shown that brood rearing and some other tasks are performed around the clock; for such activities a more apt analogy would be E. O. Wilson's "colony as a factory" (with bees working day or night shifts, perhaps). Also, bound by the strictures of this construct, the authors labour to show how the superorganism can die (when the queen dies or leaves with a swarm) while the colony lives on (with a replacement queen). Perhaps this theme, which has important implications for sociogenetics, would be better illuminated by a "colony as a corporation" analogy (the faces may change, but the brand stays the same . . .).

A second problem is that it is difficult to define what a superorganism really is, as the authors' idiosyncratic roster reveals. We read that naked mole rats might be superorganisms; but *Polistes* wasps, to which these fascinating eusocial mammals have often been compared, are not even considered. Even more baffling is the omission of *Polybia* and other swarm-founding wasps, which in some ways display honey bee-like social complexity. These problems indi-

cate that the typological perspective taken here is not the most effective one for understanding the evolution and mechanisms of colony function. An alternative approach that is proving to be productive is to invoke a heuristic metaphor to help analyse a specific feature of colony biology, such as Southwick's "colony as a homeotherm" to describe thermoregulation, or S. Camazine's "colony as an automaton" to describe nest organization.

The book does make a strong case for the idea that natural selection at the level of the colony can be a potent influence on the evolution of both individual and colony traits, an insight that originated from Darwin. It deals comprehensively with only colony biology (as was intended), and social-insect biologists will appreciate the encyclopaedic literature review. Most aspects of the biology of the individual bee are covered only superficially and important areas such as neuroethology are virtually ignored. There are reasonably balanced treatments of several controversial or

new topics, including kin recognition and the genetics of division of labour. The extensive coverage of bee genetics (Moritz's own area of expertise) is useful because this topic has received much recent empirical and theoretical attention. But because of its misinterpretations, errors of fact and attribution, and numerous typographical errors, the book will not compete with Tom Seeley's *Honey Bee Ecology* (Princeton University Press, 1985) and Mark Winston's *The Biology of the Honey Bee* (Harvard University Press, 1987) as the most useful and enjoyable treatises on the honey bee (individual and colony) for students or generalists. □

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■ Yoshiaki Itô's *Behaviour and Social Evolution of Wasps: The Communal Aggregation Hypothesis* has just been published in Oxford University Press's Series in Ecology and Evolution. The author argues that kin selection has been over-emphasized as an evolutionary explanation of sociality. £30, \$61 (hbk); £28, \$13.50 (pbk).

Forbidden symmetries

Walter Steurer

Quasicrystals: A Primer. By C. Janot. Oxford University Press: 1992. Pp. 320. £42.50, \$65.

"GOOD order is the foundation of all good things", wrote Edmund Burke. For many years, crystallographers were sure that fivefold symmetry and crystalline order were incompatible. But in 1984, D. Shechtman's discovery of grains with icosahedral diffraction symmetry in a rapidly cooled aluminium-manganese alloy opened heated debates about the existence of this new ordering state of matter. The feverish activity in the study of structure and properties of these phases, later called quasicrystals, is reflected in more than 2,000 publications so far: how noncrystallographic symmetry and quasiperiodic translational order combine to give perfectly ordered structures is now essentially understood. What quasicrystals are good for is hinted at by Janot: "For quasicrystals, the age of effectiveness has already started to overlap the dreaming period". Indeed, quasicrystals are now finding uses as surface coatings, for instance for non-stick frying pans.

An easily readable textbook to guide a wide range of readers through this enormous amount of diverse information has long been needed. In *Quasicrystals*, Janot presents clearly the fundamentals of quasicrystal research, starting with an introduction to crystallography that cov-

ers geometrical properties and construction methods of quasiperiodic tilings. In the following experimental section, he describes the preparation and characterization of real quasicrystals, and includes some wonderful photographs of quasicrystals with dodecahedral or triacontahedral growth morphology. An answer to the question "where are the atoms?" can be found in the chapters on quasicrystal structure analysis. The theory and practice of quasicrystallography's most powerful tool, the high-dimensional embedding method, is presented using the example of icosahedral Al-Cu-Li. The strange behaviour of phasons, or phase fluctuations, as well as defects and lattice dynamics, are discussed in the chapters on the physics of quasicrystals.

Janot was one of the pioneers of experimental quasicrystal research, and his authority and influence pervade the book. This sometimes results, however, in a preponderance of his own work on icosahedral quasicrystals at the cost of important contributions by other scientists. And decagonal phases, or two-dimensional quasicrystals, gain only a mention.

On the whole, however, the book is an excellent primer for all who are interested in the fascinating field of quasicrystals. □

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Primary structure and functional expression of a mouse inward rectifier potassium channel

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A complementary DNA encoding an inward rectifier K⁺ channel (IRK1) was isolated from a mouse macrophage cell line by expression cloning. This channel conducts inward K⁺ current below the K⁺ equilibrium potential but passes little outward K⁺ current. The IRK1 channel contains only two putative transmembrane segments per subunit and corresponds to the inner core structure of voltage-gated K⁺ channels. The IRK1 channel and an ATP-regulated K⁺ channel show extensive sequence similarity and constitute a new superfamily.

RECENT studies have revealed the molecular heterogeneity and diversity of voltage-gated K⁺ and calcium-activated K⁺ channels^{1,2}. In addition to these K⁺ channels, which are activated by membrane depolarization and pass outward K⁺ current, there are other types of K⁺ channels which are not gated by voltage. One example is the inward rectifier K⁺ channel³, which allows K⁺ influx but little K⁺ efflux. These K⁺ channels have been found in a variety of cell types including skeletal^{3,4} and cardiac⁵ muscle cells, starfish and tunicate oocytes^{6,7}, neurons⁸⁻¹¹, glial cells¹², blood cells^{13,14} and endothelial cells¹⁵.

The inward rectifier K⁺ channels have significant roles in maintaining the resting potential and in controlling excitability of a cell. The physiological functions of the inward rectifier K⁺ channels stem from their unique rectification property and consist of three parts¹⁶. First, the absence of outward conductance at highly depolarized membrane potentials allows a cell that expresses predominantly the inward rectifier to maintain prolonged depolarization. This is important for the generation of prolonged action potentials in heart ventricular cells, and for the prevention of double fertilization of oocytes¹⁷. Second, the large inward conductance at membrane potentials below the K⁺ equilibrium potential (E_K) prevents excessive hyperpolarization, which may be caused by the electrogenic Na⁺ pump¹⁶. Third, the slight outward conductance of inward rectifier K⁺ channels at membrane potentials just above E_K helps to keep the resting membrane potential close to E_K . Modulation of this conductance level changes the resting potential and alters the excitability of the cell. For example, it is well known that activation of a particular type of inward rectifier K⁺ channels by acetylcholine causes hyperpolarization of cardiac pacemaker cells and slows the heartbeat¹⁸.

The inward rectification properties essential for the physiological functions of these K⁺ channels have been characterized at the mechanistic level. The channels are permeable to an inward flow of K⁺ ions at membrane potentials below E_K , which may be varied by changing the extracellular K⁺ ion concentration⁶. Therefore, the inward rectifier K⁺ channels do not activate over a fixed range of membrane potential, unlike voltage-gated K⁺ channels. The inward rectification has been shown to be mainly due to the blockade of outward current by internal Mg²⁺; in the absence of Mg²⁺, inward rectifier K⁺ channels show a linear current-voltage ($I-V$) relation¹⁹⁻²¹.

The extensive interaction of the inward rectifier K⁺ channel pore with permeant ions and blocking ions has been well documented. These studies reveal that the inward rectifier K⁺ channel has a long pore with multiple binding sites; permeant ions enter

the pore in single file and interact with other ions that either permeate or block the pore^{16,22,23}. Interactions between permeant ions are responsible for the fact that K⁺ conductance of the inward rectifier does not increase linearly with K⁺ concentration⁵. Extracellular cations such as Ba²⁺ and Cs⁺ block the inward rectifier in a way that depends both on the voltage and on the time elapsed following channel activation. This suggests that these blocking ions enter the open channel pore so that they sense part of the voltage drop across the membrane⁶. The steepness of this voltage dependence of the block further indicates that there are multiple binding sites for Cs⁺ in the channel pore^{6,16}.

To elucidate the physiological functions and biophysical properties of inward rectifier K⁺ channels at the molecular level, we have cloned a cDNA encoding an inward rectifier K⁺ channel (IRK1) by functional expression in *Xenopus* oocytes. The electrophysiological properties of the IRK1 channel are characteristic of an inward rectifier K⁺ channel. We describe how the primary structure of IRK1 provides new insights of channel structure and function.

Cloning of an inward rectifier K⁺ channel

To identify a source for messenger RNA suitable for expression cloning of the inward rectifier, we isolated poly(A)⁺ RNA from various tissues (rat brain and skeletal muscle, rat and guinea-pig heart, and cultured bovine aortic endothelial cells), and from various cell lines (C2C12 and L6 (myoblast), GH₃ (pituitary), GT1-1 (neuroendocrine) and J774 (macrophage)). We injected *Xenopus* oocytes with ~50 ng of poly(A)⁺ RNA from different sources and recorded membrane currents under two-electrode voltage clamp in a 90 mM K⁺ solution, which optimizes the detection of inward rectifier K⁺ currents. Poly(A)⁺ RNA from rat brain, GT1-1 cells and J774 cells (Fig. 1a, b) induced detectable expression of an inward rectifier K⁺ current. The J774 mouse macrophage cells were chosen for subsequent experiments because the expressed current was large and highly susceptible to block by external Ba²⁺. The sensitivity of the expressed current to the block by 100 μ M external Ba²⁺ enables it to be distinguished from the variable endogenous inward rectifying currents of *Xenopus* oocytes (Fig. 1a, b), which are unaffected by 100 μ M external Ba²⁺. After size fractionation, poly(A)⁺ RNA of 4.5–5.5 kb induced the largest current (Fig. 1a, b), and was used for construction of a unidirectional cDNA library. A single clone (IRK1) isolated from this library, carrying a cDNA of 5.5 kb, was sufficient to give rise to inward rectifier K⁺ current in oocytes (Fig. 1c, d). This K⁺ current resembles the current induced by total poly(A)⁺ RNA from J774 cells in its rapid activation below E_K .

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FIG. 1 Inward rectifying currents induced in *Xenopus* oocytes by total poly(A)⁺ RNA from the J774 mouse macrophage cell line, size-fractionated RNA, and transcribed RNA from the IRK1 cDNA clone. *a, c*, Currents recorded under two-electrode voltage clamp in 90 mM K⁺ solution from oocytes injected with *a*, poly(A)⁺ RNA, 4.5–5.5 kb poly(A)⁺ RNA or water, or *c*, RNA transcribed from the IRK1 cDNA. The holding potential was –30 mV (*a*), or 0 mV (*c*). Steps to +50, +20, –10, –40, –70 and –100 mV are shown. Scale bars indicate 500 ms and 1 μ A. *b, d*, *I*–*V* curve of data in *a* and *c*, respectively. Current amplitudes just after the capacitive transient (10 ms from the beginning of the voltage step) were plotted. Symbols in *b* represent: total poly(A)⁺ RNA (▼); 4.5–5.5 kb poly(A)⁺ RNA (●); water (▽); and in *d*, IRK1 RNA (●); water (▽).

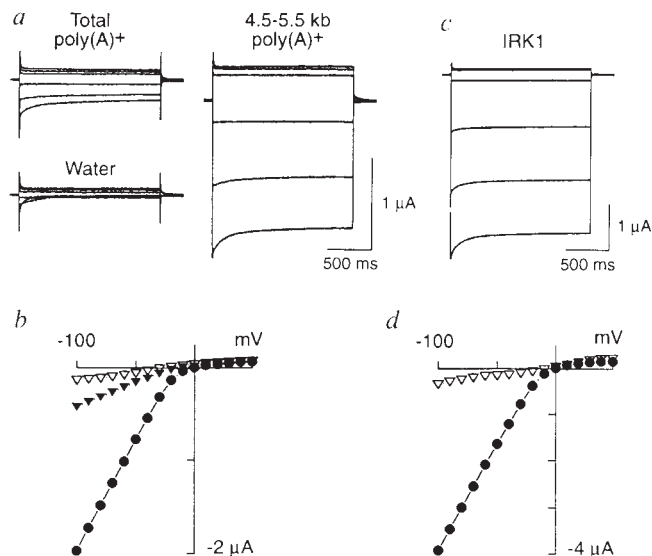
METHODS. Library construction: Poly(A)⁺ RNA was isolated from J774 mouse macrophage cells using a Fast-Track RNA isolation kit (Invitrogen) and fractionated as described⁴². Poly(A)⁺ RNA (140 μ g) was loaded onto a 5–20% sucrose gradient and centrifuged at 21,000 r.p.m. for 15.5 h at 22 °C. Thirty fractions were collected and analysed electrophysiologically. The size range of RNA in each fraction was determined by electrophoresis on a formaldehyde-agarose gel; poly(A)⁺ RNA was detected with an oligo(dT) probe on the Nytran (S&S) RNA blot. A cDNA library was constructed essentially as described⁴³. The 4.5–5.5 kb-enriched RNA fraction was reverse-transcribed using MMV Superscript (BRL) at 42 °C by priming with *Not*I-oligo(dT) primer adaptors. After ligating a *Bst*X1 adaptor and digestion with *Not*I, cDNA was size-fractionated on a potassium acetate gradient. cDNAs larger than 3 kb were ligated to a *Bst*X1–*Not*I-digested pcDNA1/Amp plasmid (Invitrogen). Recombinant plasmids were electroporated into DH10B bacteria (BRL). The initial library was composed of 120,000 independent recombinants. This primary library was size-selected further by digesting plasmids with *Not*I, selecting DNA fragments larger than 9 kb (about quarter of the total library), then religating and transforming bacteria. Sixteen pools of 5,000 recombinants were screened. RNA was transcribed from *Not*I-digested DNA using methylated cap analogue and T7 polymerase as described⁴⁴, except that the reaction products were not treated with DNase. Subdivision of a positive pool was repeated until a single clone was obtained.

Electrophysiology of the IRK1 current

The channel encoded by the IRK1 cDNA clone is an authentic inward rectifier K⁺ channel, because (1) the expressed current always activates at membrane potentials below E_K ; (2) the slope conductance does not increase linearly with external K⁺ concentration; and (3) the current is blocked by external Na⁺, Ba²⁺ and Cs⁺. The single channel conductance (21 pS) and inward rectification of the IRK1 single channel current are also characteristic of inward rectifier K⁺ channels.

The salient feature of an inward rectifier is its ability to pass large inward K⁺ current below E_K but only minimal outward K⁺ current⁶. To investigate this feature, we varied E_K by changing the K⁺ concentration of the external solution and measured the activation potential of the IRK1 current, which showed strong inward rectification (Fig. 2*a, b*). The activation potential is defined as the potential at which the slope conductance changes noticeably; the slope conductance is small at potentials more depolarized than the activation potential and begins to increase at the activation potential, reaching a high level at more hyperpolarized potentials. The activation potentials measured in three experiments were 0 ± 2 mV (90 mM K⁺), -17 ± 2 mV (45 mM K⁺), -41 ± 2 mV (20 mM K⁺), -56 ± 2 mV (10 mM K⁺) and -75 ± 3 mV (4 mM K⁺), which are in good agreement with E_K values in these solutions predicted from the Nernst equation (see legend to Fig. 2*a, b*). These results show that the IRK1 channels are K⁺ channels that activate at membrane potentials below E_K and pass inward K⁺ currents.

Another characteristic feature of inward rectifiers is the non-linear dependence of conductance on K⁺ concentration: the slope conductance varies with the square root of the external K⁺ concentration⁵. Indeed, the double-logarithmic plot of the slope conductance versus extracellular K⁺ concentration (4–20 mM) was fitted with a straight line of slope 0.47 ± 0.03 ($n = 3$). Thus the IRK1 current mimics the inward rectifier currents in its deviation from the independence principle, thereby revealing interactions between K⁺ ions as they go through the channel.



Electrophysiology: Oocytes were injected with 50 nl RNA ($1 \mu\text{g } \mu\text{l}^{-1}$), and treated with collagenase (2 mg ml^{-1}) for 2 h at room temperature. Electrophysiological recordings were carried out 48–96 h later at 22 ± 2 °C by two-electrode voltage clamp⁴⁴. Data acquisition and analysis were done on an 80386-based microcomputer using pclamp program and TL-1 A/D converter (Axon Instruments). Microelectrodes were filled with 3M KCl; the resistance was 0.7–1.4 Mohm. Bath solution contained 90 mM KCl, 3 mM MgCl₂, 5 mM HEPES (pH 7.4) (the 90 mM K⁺ solution) with 300 μM niflumic acid to block Cl[–] channels.

A third characteristic feature of inward rectifiers is their susceptibility to the block of the channel pore by extracellular cations^{4,6,24}. Such a block was indicated by a prominent inactivation of the IRK1 current in solutions containing 20, 10 (Fig. 2*a*) or 4 mM K⁺ and increased Na⁺ concentrations. Indeed, in external solutions of varying Na⁺ concentrations (*N*-methylglucamine was used to maintain the ionic strength as the Na⁺ concentration was varied, and the K⁺ concentration was fixed at 4 mM), the inactivation increased with the external Na⁺ concentration and was both voltage- and time-dependent (Fig. 2*c, d*). In addition to this Na⁺ block, the IRK1 channel was blocked in a voltage- and time-dependent way by external Ba²⁺ (Fig. 2*e, f*) or Cs⁺ (Fig. 2*g, h*), suggesting that all three ions act as open channel pore blockers. The steep voltage dependence of Cs⁺ block was quantitated by first plotting the ratios of steady-state current levels in the presence and absence of Cs⁺ as a function of external Cs⁺ concentration (to obtain K_i for the Cs⁺ block at each membrane potential), and then fitting the double logarithmic plot of K_i versus membrane potential with a straight line. A tenfold change in K_i corresponded to a change in membrane potential of 38.1 mV. This implies that the fractional distance of the Cs⁺ binding site in the membrane electric field is 1.53, if we assume that only one Cs⁺ ion enters the pore and blocks K⁺ permeation. This apparent anomaly indicates that several Cs⁺ ions reside in a single channel pore, as shown previously for inward rectifiers^{6,16}.

By demonstrating K⁺ selectivity, inward rectification and interaction between permeant ions and blocking ions, our results show that the IRK1 channel is an inward rectifier K⁺ channel. Apart from the inward rectifier K⁺ channels, cardiac muscarinic K⁺ channels and ATP-sensitive K⁺ channels also show moderate inward rectification^{25–27}. The outward K⁺ conductances of these two types of channels are much larger than that of the IRK1 channel. In addition, the single channel conductance of IRK1 channels in cell-attached patches was 21 ± 2 pS ($n = 4$) (140 mM external K⁺/~90 mM intracellular K⁺, at 21 ± 2 °C) (Fig. 3*a, b*), which is similar to that of inward rectifiers (20–30 pS)^{13,21} but

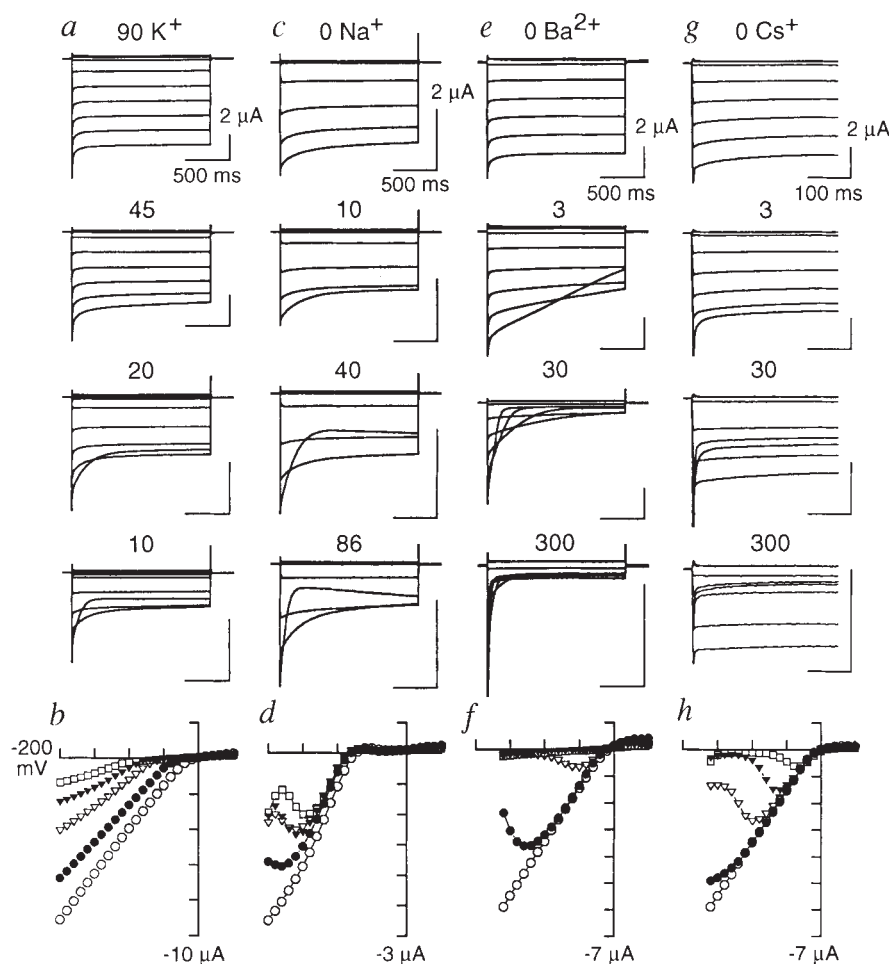
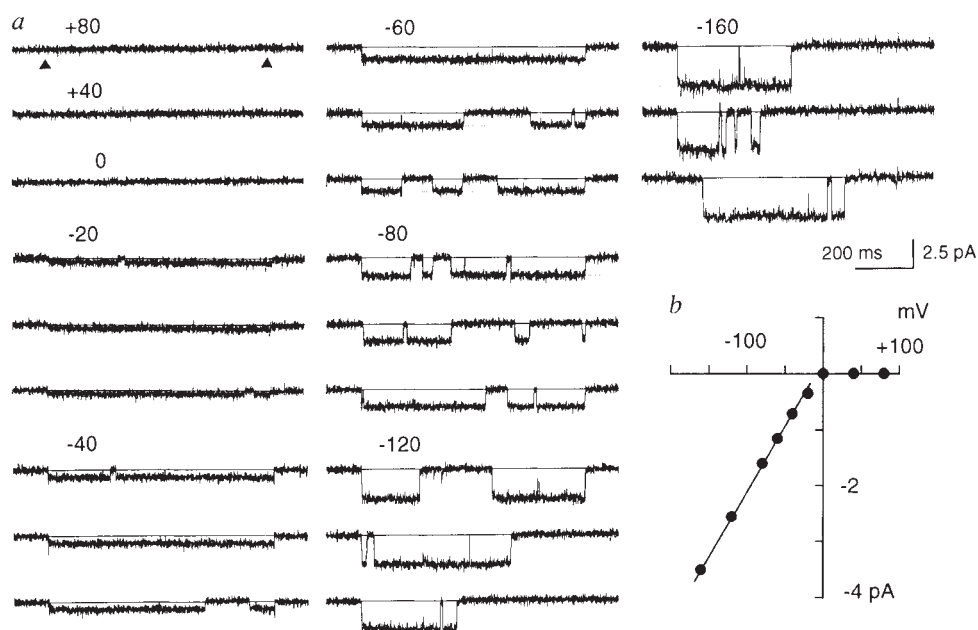


FIG. 2 The IRK1 K^+ current exhibits characteristic inward rectification properties and open channel block by external Na^+ , Ba^{2+} and Cs^+ . *a, c, e* and *g*, Current recordings under voltage clamp. Holding potential was 0 mV. Traces elicited by steps to +50, +20, -10, -40, -70, -100, -130 and -160 mV are shown. An additional trace elicited by hyperpolarization to -190 mV is shown in *a* and *c*. Scale bars indicate 500 ms and 2 μA (*a, c* and *e*), 100 ms and 2 μA (*g*). Voltage- and time-dependent block of the IRK1 current is evident in 40 and 86 mM Na^+ (*c*), 30 μM Ba^{2+} (*e*) and 30 μM Cs^+ (*g*). *b, d, f* and *h*, *I-V* relations of *a, c, e* and *g*, respectively. Current amplitudes just after the capacitive transient (10 ms from the start of voltage pulses) are plotted in *b*, and current amplitudes at the end of the 1,600-ms voltage pulses are plotted in *d, f* and *h*. *a, b*, Current recordings in solutions of 90 mM K^+ ($\circ$), 45 mM K^+ ($\bullet$), 20 mM K^+ (∇), 10 mM K^+ ($\blacktriangledown$) and 4 mM K^+ ($\square$) (data not shown in *a*). K^+ was substituted with Na^+ . E_K values in these solutions, as predicted by the Nernst equation, were: 0 mV (90 mM K^+), -17.4 mV (45 mM K^+), -37.9 mV (20 mM K^+), -55.3 mV (10 mM K^+) and -78.4 mV (4 mM K^+). (The intracellular K^+ concentration of oocytes was assumed to be 90 mM as before⁴⁵). IRK1 current in 90 mM K^+ solution showed slight inactivation shortly after channel activation (within 200 ms), which could be due to block by certain extracellular ions such as Mg^{2+} (ref. 46). *c, d*, Current recordings in solutions of 0 mM K^+ ($\circ$), 10 mM K^+ ($\bullet$), 20 mM K^+ (∇) (data not shown in *c*), 40 mM K^+ ($\blacktriangledown$) and 86 mM Na^+ ($\square$) solution. All solutions contained 4 mM K^+ , 3 mM Mg^{2+} , 5 mM HEPES (pH 7.4). Na^+ was replaced with *N*-methylglucamine. The sum of Na^+ and *N*-methylglucamine was 86 mM. *e, f*, Current recordings in solutions of 0 μM ($\circ$), 3 μM ($\bullet$), 30 μM (∇), 300 μM ($\blacktriangledown$) and 3 mM Ba^{2+} ($\square$) (data not shown in *e*). $BaCl_2$ was added to the 90 mM K^+ solution without correcting the ionic strength. *g, h*, Current recordings in solutions of 0 μM ($\circ$), 3 μM ($\bullet$), 30 μM (∇), 300 μM ($\blacktriangledown$) and 3 mM Cs^+ ($\square$) (data not shown in *g*). $CsCl$ was added to the 90 mM K^+ solution without correcting the ionic strength.

FIG. 3 Single-channel activities of the IRK1 K^+ channel show inward rectification. *a*, Current recordings of single channel activities elicited by voltage steps to various potentials from 0 mV in the cell-attached mode. The linear leakage and capacitive components were subtracted using averaged blank records. At -40 mV, as there were no blank traces recorded, records at +40 mV were used as leak templates. The potentials shown represent the pipette potential, the difference between the membrane potential and the pipette potential is the resting potential of the oocyte, which could range between 0 and -10 mV in the high K^+ solution. Scale bars indicate 200 ms and 2.5 pA. Triangles show beginning and end of voltage steps. *b*, *I-V* plot of the single channel current in *a*. *V* axis shows the pipette potential; the straight line was fitted by eye. Slope conductance, 23 pS.

METHODS. Single-channel recordings were made in the cell-attached or the inside-out excised patch configuration of the patch clamp, using an EPC7 amplifier (List) at 22–25 °C. The pipette solution was 140 mM KCl, 3 mM $MgCl_2$, 5 mM HEPES (pH 7.4). The bath solution was 110 mM KCl, 10 mM $MgCl_2$, 2 mM K_2 ATP, 5 mM EDTA, 5 mM HEPES, 25 mM KOH (pH 7.2). Total K^+ concentration was 140 mM, and the free Mg^{2+} concentration was calculated to be 3 mM. Data were filtered at



1 kHz by an 8-pole bessel filter, sampled at 2–8 kHz through an A/D converter TL1, and stored in an 80386-based computer. Single-channel current amplitudes were determined by fitting lines to recordings at each potential. The slope conductance was determined from a straight line fitted by eye.

FIG. 4 The primary structure of the IRK1 inward rectifier K^+ channel. **a**, Nucleotide and deduced amino-acid sequence of IRK1. The IRK1 cDNA was sequenced on both strands using Sequenase (USB). The deduced amino-acid sequence is displayed above the nucleotide sequence of the coding strand of the IRK1 cDNA. Nucleotide A of the initiation codon (ATG) is assigned as +1. The IRK1 clone is ~5.5 kb long and has only one long open reading frame which predicts a protein of 428 amino acids. Only part of the 3' untranslated sequence is shown. The potential transmembrane segments M1 and M2 are boxed. Also boxed is a segment with sequence similarity to the H5 region of the voltage-gated K^+ channel. There is one potential cAMP and cGMP-dependent protein kinase phosphorylation site (S426), four potential protein kinase C phosphorylation sites (S3, T6, S357, T383), and two potential tyrosine kinase C phosphorylation sites (Y242, Y366). **b**, Alignment of amino-acid sequences of IRK1 and the ATP-regulated K^+ channel (ROMK1)³³. Vertical lines indicate amino-acid identity and stops indicate amino-acid similarity. Amino acids in the following groups were classified as similar (one-letter code): A, S and T; D and E, N and Q; R and K; I, L, M and V; F, Y and W. Dashes indicate gaps introduced into the sequence to improve alignment. The proposed transmembrane regions M1 and M2 and the H5 like segments are boxed. The ATP-binding site in ROMK1 is doubly underlined. By use of Monte Carlo and Needleman-Wunch statistical analysis, these two sequences were significantly related. No other sequences with significant similarity to the IRK1 amino-acid sequence were detected in the GenBank or EMBL data bases. **c-e**, Alignments of the IRK1 and other K^+ channel sequences in the H5 (**c**), S5 (**d**) and S6 (**e**) region. Boxed residues are identical to those of the IRK1 at the corresponding positions. The K^+ channels included in this alignments are: voltage-gated K^+ channels of the Shaker subfamily (Kv1.1/RCK1)⁴⁷, the Shab subfamily (Kv2.1/drK1)⁴⁸, the Shaw subfamily (Kv3.1/NGK2)⁴⁹ and the Shal subfamily (Kv4.1/mouse Shal)⁵⁰, a Ca^{2+} -dependent K^+ channel (Slo)⁵¹ and a plant K^+ channel (KAT1)⁵².

and skeletal muscle, but not in kidney. The abundance of this 5.5-kb mRNA was much higher in skeletal muscle, heart and forebrain than in cerebellum.

Discussion

We have cloned a cDNA encoding an inward rectifier K^+ channel (IRK1) by functional expression. Expression in *Xenopus* oocytes results in a K^+ current with electrophysiological properties characteristic of the inward rectifier. Although the IRK1 cDNA was isolated from the mouse macrophage cell line J774, mRNA encoding IRK1 is also detected in brain, heart, and skeletal muscle, all of which are known to express inward rectifiers with properties similar to those of the IRK1 channel.

The primary structure of IRK1 is of particular interest with regard to both channel structure and evolution. The IRK1 channel polypeptide contains two hydrophobic segments which may be aligned with the S5 and S6 segments of voltage-gated K^+ channels, as well as a sequence in between the two hydrophobic segments that shows extensive similarity with the H5 sequence. This suggests that these sequences harbour most or all of the structure that line a K^+ channel pore. The IRK1 channel polypeptide, however, does not contain any other hydrophobic segments that would correspond to S1, S2 and S3 of the voltage-gated channels (Fig. 6). The S1, S2 and S3 sequences contain regularly spaced hydrophobic residues that are not conserved within each subfamily of voltage-gated K^+ channels, indicating that these segments may form transmembrane α -helices with the non-conserved hydrophobic residues facing lipid molecules of the membrane³⁵, rather as in the photosynthetic reaction centre³⁶. We propose that the inward rectifier contains only the

b

IRK1	MGSVRTNRYISIVSSEEDGMK LATMAVANGFGNGKSKVH-----	38
ROMK1	MGA-----SERSVFRVLIRALTERMFK	22
IRK1	-----TRQQCRSRFVKKDGRCNVQFINVGEGQ- RYLADIPT	75
ROMK1	HLRRWFITHIFGRSRQ--RARLVSKEGRCNIEFGNVDAQSRFIFVVDIWT	70
IRK1	TCVDIRWRWMLVIFCLAFVLSWLFPGCVFWLIALLHGDL-----DTSK	117
ROMK1	TVLDLKWRYKMTVFITAFVLSWLFPGCVFWLIALLHGDLPEFYPPDNRT-	119
IRK1	VSKACVSEVNSFTAFLFSLIETQTTIGYGFRCVTDECPITAVFMVVFQSI	167
ROMK1	---PCVENINGMTSAFLFSLIETQTTIGYGFRFVTEQCATAIFLLIFQSI	166
IRK1	GCIIDAFIIGA VMAMAKPKKRNETLVFSHNAVIA MRDGLCLMWRVGNL	217
ROMK1	GVIINSFMCGA ILAKISRPKKRAKTITFSKNAVISKRGKLC LLIRVANL	216
IRK1	RKSHLVEAHVRAQLLKSRTSEGEYIPLDQIDINVGFDSGIDRIFLVSP	267
ROMK1	RKSLLI GSHIYGKLLKTTITPEGETIILDQTNIN FVVDAGNENLFFISPL	266
IRK1	TIVHEIDEDSPLYDLKSKQDIDNADFEIVVILEGMVEATAMTTQCRSSYLA	317
ROMK1	TIYHIIDHNSPFFHMAAETLSQQDFELVVFVLDGTVESTSATCQVRTSYVP	316
IRK1	NEILWGHRYEPVLF---EEKHYIKVDYSRFHKTYEV-----	350
ROMK1	EEVLWGYRFVPIVSKTKEGK--YRVDPHNFGKTVEVETPHCAMCLYNEKD	364
IRK1	-----PNTPLCSARDLAEKKYILSNANSFCYENEVALTSKEEEE	389
ROMK1	ARARMKRGYDNP-----FVLSEVDETD	387
IRK1	DSENGVPSTSTDSPPGIDLHNQASVPLEPRPLRRESEI	428
ROMK1	DTQM	391

c

	H 5
IRK1	AFLFSIETQTTIGYGFR
Kv1.1	AFWWAVVSMTTVGYGDM
Kv2.1	SFWWATITMTTVGYGDI
Kv3.1	GFWWAVVTMTTLGYGDM
Kv4.1	AFWYTIIVTMTTLGYGDM
Slo	CVYFLIVTMTTLGYGDV
KAT1	ALYWSITTLTTLGYGDF
	H 5

d

	M 1
IRK1	IFCLAFVLSWLFPGCVFWLIAL
Kv1.1	LGLLIFFLFVIGVILFSSAVYFA
Kv2.1	LGLLILFLAMGIMIFSSLVFFA
Kv3.1	FLLLIIFLALGLVILFATMIYFA
Kv4.1	LGFLFLSLTMAIIIFATVMFYA
Slo	LAQLVLSIFISVWLTAAGIIHL
KAT1	CTKLISVTLFAIHCAGCFNYLI
	S 5

e

	M 2
IRK1	P[IAVFMVVFQSI]VGCITIDAFIIGA
Kv1.1	GKIVGSLCAIAGVLTIALPVPVIV
Kv2.1	GKIVGGLCCIAGVLTIALPIPIIV
Kv3.1	GMLVGALCALAGVLTIAIMPVPVIV
Kv4.1	GKIFGSI CSLGVLTIALPVPVIV
Slo	GRTFLVFFLLVGLAMFASSIPEII
KAT1	D[IFFMM]FNGLGLTAYLIIGNMTNLVV
	S 6

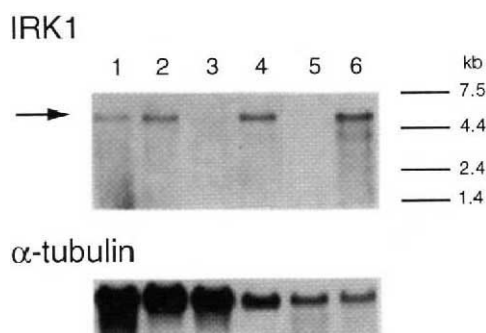


FIG. 5 Distribution of IRK1 mRNA in tissues. Upper panel, northern blot analysis of IRK1 mRNA expression. Lanes represent poly(A)⁺ RNA from (1) J774 cells, (2) mouse forebrain, (3) cerebellum, (4) heart, (5) kidney and (6) leg skeletal muscle. Forebrain includes cerebral cortex, hippocampus, basal ganglia, thalamus, hypothalamus and olfactory bulb. The migration positions of RNA size markers are shown on the right. A major RNA band of 5.5 kb that hybridizes to the IRK1 cDNA is indicated by an arrow. Lower panel, to control for the integrity of RNA, the same blot was reprobbed with a probe for α -tubulin, which hybridized to a 1.6-kb RNA.

METHODS. Poly(A)⁺ RNA was isolated using a Fast-Track RNA isolation kit (Invitrogen) from tissues of 10-week-old male BALB/c mice, from which the J774 cell line was established. RNA sample concentrations were determined by absorbance at 260 nm, and 3 μ g poly(A)⁺ RNA was fractionated on a 0.7% agarose-formaldehyde gel and transferred to a nylon membrane. A 3.7-kb *Bst*X1-*Not*I fragment from the 3' end of IRK1 was labelled with ³²P by random priming. Hybridization was done as described⁴⁴, filters were washed with 0.1 $\times$ SSC, 1.0% SDS at 65 $^{\circ}$ C for 15 min and autoradiographed. An RNA size marker (BRL) was probed separately with ³²P-labelled λ DNA.

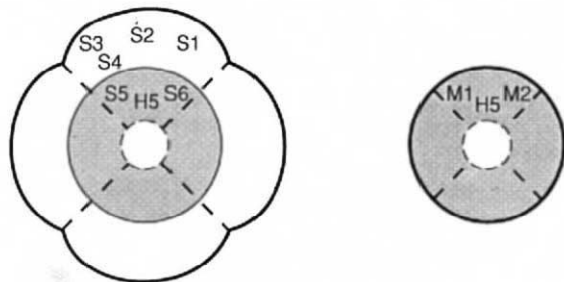
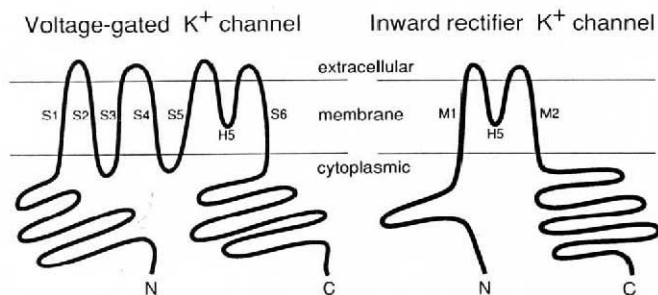


FIG. 6 Proposed membrane topology of the IRK1 inward rectifier K⁺ channel. By analogy with the suggested membrane arrangement of the voltage-gated K⁺ channels, the M1 and M2 segments of the IRK1 channel are shown as membrane-spanning and the H5 sequence in between extends from the extracellular surface into the membrane. A similar topology has been proposed for the ATP-regulated K⁺ channel³³. Compared with the voltage-gated K⁺ channel, which may contain an outer shell of S1, S2 and S3 segments in contact with the lipid environment³⁵, the inward rectifier K⁺ channel represents a more reduced structure corresponding to the inner core of the voltage-gated K⁺ channel.

inner core structure (S5, H5 and S6) necessary for K⁺ selective permeation, whereas the voltage-gated K⁺ channels contain in addition an outer shell composed of the S1, S2 and S3 segments, which may provide an electrostatic shield between the hydrophobic membrane interior and the highly charged S4 sequence that is likely to be a membrane-spanning voltage sensor^{16,37-40} (Fig. 6).

The extensive sequence similarity between the IRK1 inward rectifier K⁺ channel and the ATP-regulated K⁺ channel³³ suggests that these channels belong to a new superfamily of K⁺ channels which are related to, but distinct from, voltage-gated K⁺ channels. This new superfamily will probably include members that correspond to other electrophysiologically and pharmacologically distinct inward rectifier K⁺ channels⁶, ATP-sensitive K⁺ channels⁴¹ and possibly the muscarinic K⁺ channels^{25,26}. The many physiological functions of this group of K⁺ channels include control of neuronal excitability¹⁰, control of pancreatic release of insulin⁴¹, and cholinergic regulation of heartbeat¹⁸.

The inward rectifier K⁺ channel is a classic example of the highly selective K⁺ channel with a long pore and multiple K⁺ binding sites, partly because the pore of the inward rectifier interacts strongly not only with permeant ions but also with blocking ions such as Na⁺, Cs⁺ and Ba²⁺ from the extracellular side, and Mg²⁺ from the cytoplasmic side. The voltage- and time-dependent block of IRK1 currents by extracellular Na⁺, Ba²⁺ and Cs⁺ suggests that these ions act as open channel pore blockers. The steep voltage dependence of Cs⁺ block further indicates that a single IRK1 channel pore contains multiple binding sites for Cs⁺. The effects of internal Mg²⁺ on IRK1 channels expressed in *Xenopus* oocytes are difficult to analyse because the IRK1 channels in excised patches stop conducting within minutes. Nevertheless, we have found that the activation potential of the IRK1 K⁺ current closely approximates the K⁺ equilibrium potential (E_K) over a wide range of external K⁺ concentrations (Fig. 2a, b). This indicates that the IRK1 channel does not permit substantial outward K⁺ flux because its pore is blocked by internal cations. Previous studies have implicated the H5 segment as part of the pre-lining structure of voltage-gated K⁺ channels³⁴. Further mutagenesis of the H5 and other sequences of IRK1 will help to elucidate the mechanisms for K⁺ permeation, the open channel block by various ions, and the inward rectification that operate under physiological conditions and which contribute to the gating of inward rectifier K⁺ channels. □

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LETTERS TO NATURE

The Guitar nebula: a bow shock from a slow-spin, high-velocity neutron star

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THE observable interactions of neutron stars with their local environment provide an opportunity for measuring the properties of both relativistic pulsar winds and the surrounding interstellar medium. Here we report the discovery of a prominent nebula produced by the motion of a high-velocity pulsar, PSR 2224+65, through partially neutral gas. The pulsar's transverse speed of $\geq 800 \text{ km s}^{-1}$ makes it arguably the fastest known star in the Galaxy and guarantees that it will ultimately escape the galactic potential well. A deep H α image reveals a bright head and a faint, limb-brightened 'body' whose variable width suggests that the ambient interstellar gas has density variations on length scales $\leq 0.1 \text{ pc}$. Thermalization of shock energy occurs at a rate of about 10^{-2} times the pulsar's spindown loss rate. Our observations provide some insights into the likelihood of finding shocks around other pulsars and the use of nebulae to find high-velocity neutron stars either not acting as pulsars or with their radiation beamed away from the Earth.

Recent work has highlighted the variety of ways that radio pulsars affect their environment through their spindown-generated winds. Several pulsars with large rotational energy loss rates show shock structures or synchrotron nebulae associated with the relativistic pulsar wind, including the Crab^{1–3} and Vela⁴ pulsars, PSR 0540–69 in the LMC, PSR 1951+32 in the CTB80 supernova remnant⁵, and PSR 1757–24, a high velocity pulsar which is outrunning the supernova shell which used to surround it^{6,7}. These objects all have energy loss rates $\dot{E} = I\Omega\dot{\Omega}$ in excess of $10^{36} \text{ erg s}^{-1}$. The millisecond pulsar 1957+20 ($10^{35.2} \text{ erg s}^{-1}$) induces a beam-heated wind from its eclipsing companion and it has created a limb-brightened H α shock whose standoff distance results from a balance between the pulsar wind and the ram pressure from the interstellar medium (ISM)⁸. The observed range of structures depends on the spindown luminosity and velocity of the pulsar, on the nature of the surrounding medium and on the carriers of the spindown luminosity. The energy spectrum of the pulsar wind particles has observable effects on wind termination structures³ and on the radiation of X-rays from post-shock regions⁹.

Here we report the discovery of a bow shock from a pulsar with a spindown luminosity more than 100 times smaller than the examples above. PSR 2224+65 has quite ordinary spin parameters: $P = 0.68 \text{ s}$, $\dot{P} = 9.7 \times 10^{-15} \text{ s s}^{-1}$, $\dot{E} = 10^{33.1} \text{ erg s}^{-1}$.

The bow shock is similar to that of PSR 1957+20 in that it arises from interaction with the general ISM but it is evident (in spite of the small $\dot{E}$) because of the very large space velocity.

Observations in the summer of 1992 used a CCD camera and spectrograph (the Four Shooter¹⁰) on the Hale 5-m telescope at Palomar. Our programme focused on high-velocity objects within 2 kpc of the Sun that were otherwise unconstrained with respect to spindown properties. PSR 2224+65 was a notable member of our source list because it showed a large space velocity as determined from interstellar scintillations¹¹, with apparent corroboration from interferometry¹². Images were obtained with narrow-band (20 Å at 6564 Å) and wide-band (150 Å) H α filters. The Four Shooter's spectrograph was used to constrain the excitation of shocked gas and to estimate radial velocities of nebular components.

Figure 1 shows the narrow-band H α image (with exposure totalling 5,700 s) obtained on 31 July. A cross designates the pulsar position and the head of the bright part of the bow shock.

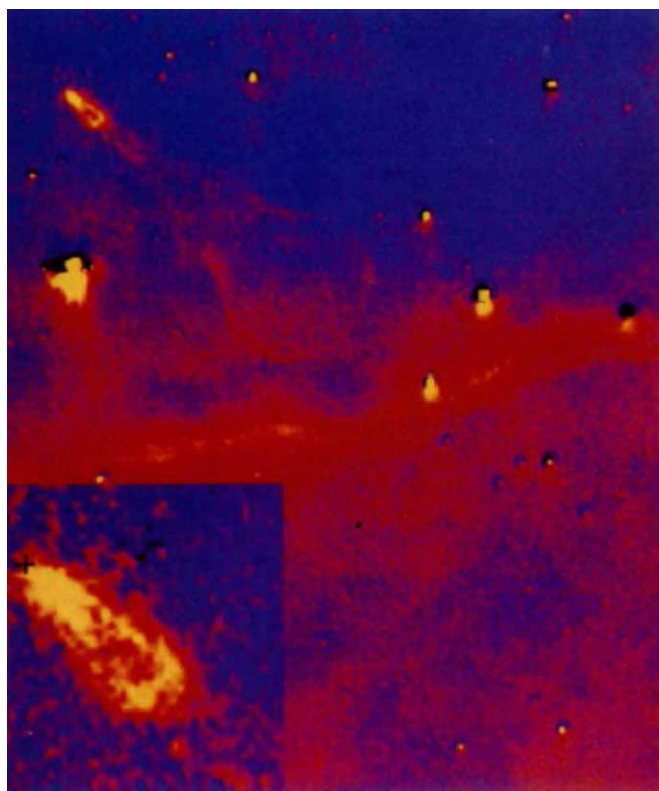


FIG. 1 H α image showing a bow-shock nebula produced by the high-velocity pulsar 2224+65. North is upward and east to the left. The width of the figure is 2.7 arcmin. The inset shows detail of the bright head, with a cross indicating the pulsar position during the epoch of the observations. The inset is 17 arcsec on a side and is at $\times 4$ magnification. The pseudocolour scale represents the most intense features as yellow, the weakest as black.

The pulsar direction and axis of the shock are to the northeast. A bright filament or arc, not directly related to the pulsar shock, runs nearly east-west, curving slightly to the northwest. The pulsar position was determined from very large array (VLA) observations (J.M.C., T. H. Hankins, J. M. Weisberg and R. J. Dewey, unpublished data) made in 1985, 1986 and 1990, which indicate a position in 1992.6 of right ascension = 22 h 24 min 17.48 (± 0.03) s, declination = $65^\circ 20' 16.85$ (± 0.13) s in 1950 coordinates and proper motion $\mu = 0.175 \pm 0.04$ arcsec yr $^{-1}$ at position angle $54^\circ \pm 12^\circ$. The direction of the pulsar's proper motion coincides with the axis of the shock nebula, within the errors. Alignment of the optical and radio reference frames, good to about 0.5 arcsec (< 1.5 pixel), indicates that the pulsar resides ≈ 0.5 arcsec from the bow shock termination point. The dispersion measure ($DM = \int_0^D ds n_e = 35.3$ pc cm $^{-3}$) of the pulsar, and an improved model for the galactic distribution of free electrons¹³ yield a distance $D \approx 1.95$ kpc, at which the transverse speed is $V_\perp = D\mu \approx 1,600 \pm 370$ km s $^{-1}$. While the pulsar resides towards the outer galaxy and slightly out of the galactic plane ($l = 108.6^\circ$, $b = 6.8^\circ$), its environment displays considerable evidence for ionized material that might bias the DM upwards. In the following, we adopt a fiducial distance of 1 kpc, keeping in mind that the true distance is likely to be 1–2 kpc.

If the pulsar is as old as its timing age, $P/2\dot{P} \approx 10^6$ yr, it has moved $\sim 50^\circ$ across the sky, or $\sim 0.9 D_{\text{kpc}}$ kpc, since its birth and in a direction nearly parallel to the galactic plane (within $14^\circ \pm 12^\circ$), consistent with its present small galactic latitude. Selection against high velocities perpendicular to the plane appears to have taken place in the low-latitude search that discovered PSR 2224 + 65. The large velocity ≥ 800 km s $^{-1}$ is unattainable through disruption of a circular orbit by a symmetric supernova explosion, even if the companion object were a black hole. PSR 2224 + 65 therefore represents direct evidence that an explosion asymmetry provides the requisite velocity kick¹⁴.

The spectrum of the shock head (Fig. 2) shows only H α , H β lines and remnants of telluric lines after sky subtraction. The absence of metal lines is in contrast to the bright arc, which shows [O I/S III], [N II], and [S II] emission as expected for a radiative shock¹⁵. The observed Balmer decrement of the pulsar shock is $I(\text{H}\alpha)/I(\text{H}\beta) = 5.5 \pm 0.5$. The absence of lines from other elements indicates that the shock may be nonradiative¹⁶. Assuming an intrinsic decrement of three, the visual extinction $A_v \approx 1.65 \pm 0.15$, consistent with the estimated pulsar distance.

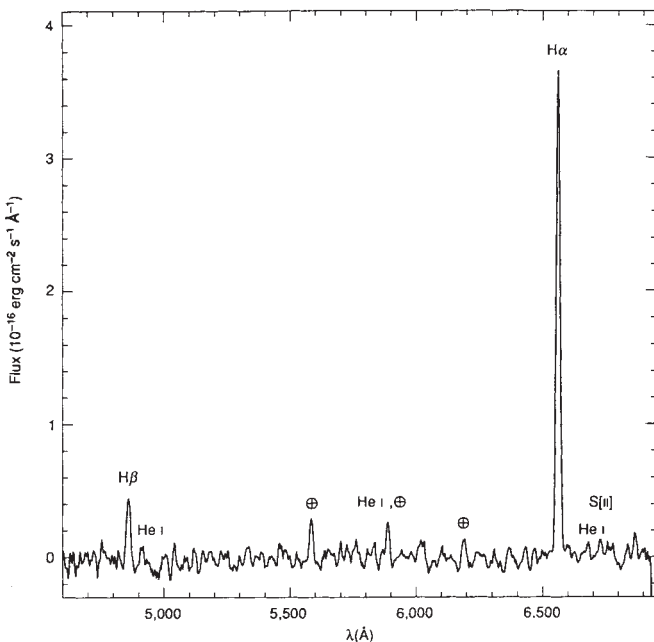


FIG. 2 Spectra of the bright head of the pulsar wind nebula. Emission lines, other than Balmer emission and telluric artefacts ($\oplus$), are undetected.

Our higher resolution spectroscopy (~ 5 Å) was not sensitive enough to distinguish the expected broad H α component (in the postshock gas) from the bright, narrow line emitted by the nebula head at the local ISM velocity. Greater sensitivity and resolution will be needed to measure the broad-component line profile and thus unravel the three-dimensional kinematics of the pulsar motion and shock expansion.

Morphology of the faint part of the nebula (the 'guitar' body) consists of a symmetric variation in width transverse to the shock axis, appearing as a closed bubble at the furthest visible point from the pulsar. Merged with this bubble is a factor-of-two smaller bubble that itself merges into a narrow neck, leading to the pulsar. Though episodic or periodic variations in $\dot{E}$ might be imagined, the simplest explanation is that the shape of the nebula results from gradients in the ambient interstellar gas density that are largely parallel to the motion of the pulsar. The closed bubble then arises from the pressure of the uncooled pulsar wind against a relatively dense wall of cool gas adjacent to the bright arc, penetrated some 10^3 yr ago. Alternatively, the closed-off appearance may result from the orientation of a truly open structure. The limb-brightened intensity varies on scales $\leq 10''$, or ~ 0.05 pc at 1 kpc distance. The bright region of the wind nebula shows where the pulsar entered a patch with factor-of-ten greater density.

The scale of the shock at the point closest to the pulsar is defined by the balance between the outflow of energy from the pulsar and ram pressure. If the pulsar wind is relativistic, consistent with the bow shock from PSR 1957 + 20 (refs 8, 17), the standoff distance of the contact discontinuity is

$$r_0 = (\dot{E}/4\pi\rho v_p^2 c)^{1/2} \quad (1)$$

where ρ is the total ambient mass density and v_p is the pulsar velocity. For PSR 2224 + 65, $r_0 \approx 29$ ($n_H v_8^2$) $^{-1/2}$ astronomical units (AU), with $v_8 = v_p/(10^8 \text{ cm s}^{-1})$ and $n_H = \rho/m_p$, about 100 times smaller than for PSR 1957 + 20 and unresolvable. Upstream from this contact discontinuity, thermal pressure in the postshock gas will hold off the bow shock in the ISM by a distance $\sim 0.35 r_0$ (ref. 17). H α emission arises from a thin shell (about one collision mean free path thick) of hydrogen atoms just inward from the bow shock, requiring a significant neutral component to pass through the bow wave. Neutral atoms exist at the small bow shock distance from the neutron star, in spite of a large and ionizing photon flux from the hot ($\sim 10^5$ K) star surface, because the photon mean free path is larger than the nebula. Ionizing emission from post-shock gas exceeds that from the neutron star, but the long recombination length $l_{\text{rec}} \sim 3 \times 10^{20} v_8/n$ cm ensures that most of the radiation will be emitted well downstream from the shock nose. The nose standoff and the down-stream cooling should provide good targets for examination by the Hubble Space Telescope and the Rosat satellite.

H α fluxes are $F_\alpha \sim 3.4 \times 10^{-4}$ and 1.1×10^{-3} photons cm $^{-2}$ s $^{-1}$ from the shock head's apex and total head, respectively. The surface brightness from the limb-brightened guitar body is about a factor of ten smaller than the average for the head. Recognizing that H α photons are produced by collisional excitation of atoms before ionization, the observed line strength is¹⁵

$$F_\alpha = (q_{\text{ex}}/q_i) n_H v_s \Delta\Omega/4\pi g(A_{\text{H}\alpha}) \quad (2)$$

where $g(A_{\text{H}\alpha}) \approx 3$ is the extinction correction, $q_{\text{ex}}/q_i \approx 0.2$ H α photons per neutral atom, n_H the ambient neutral density, $v_s \approx v_p$ the shock speed, and $\Delta\Omega$ the source solid angle that would be seen with the pulsar velocity directed at Earth. The total particle flux into the apex is (using $X = n_H/n$ as the fraction of neutral atoms) $F_p = 4\pi D^2 (X q_{\text{ex}}/q_i)^{-1} g(A_{\text{H}\alpha}) F_\alpha \approx 6 \times 10^{41} D_{\text{kpc}}^2/X$ s $^{-1}$. With a bow shock head size ranging from $\sim 1.5''$ at the apex to $\sim 4''$ at the end of the bright zone, the local mean H I density is roughly $n_H v_8 X \approx 10$ cm $^{-3}$. Variations in the dispersion measure of the pulsar over several years may yield a direct probe of the local interstellar gas density. Atoms entering the sides of the bow shock head region thermalize most of the relative

kinetic energy, giving a power $\dot{E}_{\text{particles}} \approx F_p (\frac{1}{2} m_p v^2)_{\text{particles}} \approx 10^{31.5} D_{\text{kpc}}^4 X^{-1} \text{ erg s}^{-1}$, where the typical particle shock velocity is $\sim 0.1 v_p$ from the opening angle of the shock in the head region.

The ability to observe similar shocks from active or dead pulsars is decided by the object's $\dot{E}$, space velocity, and environment. Using equation (2) and solid angle $\Delta\Omega \sim (\eta r_0/D)^2$, where $\eta \approx 30 v_p$ is a geometric factor and ignoring extinction, the H α flux is

$$F_\alpha = 10^{-3.6} \frac{v_p X \dot{E}_{33}}{D_{\text{kpc}}^2} \text{ photons cm}^{-2} \text{ s}^{-1} \quad (3)$$

a relation that fits the nebulae from both PSR 1957+20 and PSR 2224+65. The geometric factor was derived by noting that the overall dimensions of the bow shock, both parallel and transverse to the pulsar direction, scale with pulsar velocity. The flux is independent of the ambient density but vanishes as the neutral fraction $X \rightarrow 0$. Equation (3) indicates that a smaller spindown loss rate $\dot{E}$ can be compensated for by a larger pulsar velocity.

Of the $\sim 10^5$ active pulsars in the Galaxy¹⁸ $\sim 25\%$ are within one gas scale height of the galactic plane and $\sim 20\%$ of these are within warm neutral gas capable of being shock excited. This indicates that 5,000 pulsars may be able to excite nebulae. About 50 of these nebulae should reside within 1 kpc of the Sun, of which ~ 10 should be active pulsars that beam toward us. A search for shocks would require coverage of $\sim 100 \text{ deg}^2$ per object, which is not easy with present sensitivities. X-ray surveys (for example, from Rosat) may reveal nebulae, however.

An association between a long-period pulsar and an old supernova remnant

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THE birth of neutron stars is expected to be accompanied by supernovae of types Ib and II (which constitute the majority). But only a few associations between neutron stars and supernova remnants are known¹; most involve young pulsars with short periods. Older supernova remnants should also be accompanied by pulsars, but these would be less luminous and, because they would have spun down to longer periods, they would beam to a much smaller fraction of the sky². This may explain why no associations of this sort have been detected previously³. Here we report a possible such association: between pulsar 2334+61, with a relatively long period of 0.5 s, and G114.3+0.3, a fairly old supernova remnant. The flat spectral index of -0.36 ± 0.03 (refs 4, 5) and large fractional polarization suggest that the radio emission is powered by the pulsar. If so, the pulsar must have been born with a relatively short period of less than 100 ms. As the remnant is not particularly unusual morphologically, there might be many more such remnants containing pulsars that are not beamed towards us.

Of the dozen known associations between supernova remnants (SNR) and neutron stars³, most involve young pulsars. This is understandable, because (1) young pulsars are more luminous than old pulsars in most electromagnetic bands and (2) the beaming factor, the fraction of sky illuminated by the

Similarly, nebulae may be observed from some of the $\sim 10^8$ neutron stars in the Galaxy that are not active radio pulsars if their $\dot{E}$ s are not significantly smaller than those of active pulsars. Thus discovery of shock nebulae might in principle lead to identification of such dead neutron stars in the vicinity of the Solar System. $\square$

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TABLE 1 PSR 2334 + 61/G114.3 + 0.3 association

PSR 2334 + 61	SNR G114.3 + 0.3	
Period (s)	0.495	Size (arcmin) 50
Period derivative (s s ⁻¹)	1.9×10^{-13}	Flux at 1.4 GHz (Jy) 5.3
Dispersion measure (cm ⁻³ pc)	57.6	
Characteristic age, τ_c (yr)	4.1×10^4	
Rotational loss (erg s ⁻¹)	6.2×10^{34}	
$\alpha(1950)^*$	23 h 34 min 45 s	23 h 34 min 45 s
$\delta(1950)$	61° 34' 25"	61° 38' 00"
l	114.28°	114.30°
b	0.23°	0.29°
Distance (kpc)	2.5 ± 0.5	1.8 ± 0.3

* Note that $\alpha(1950)$ of the intensity centroid of G114.3 + 0.3 in Table 5 of ref. 2 is incorrect.

pulsar beam, is probably almost unity for short-period ($P \leq 0.1$ s; usually young) pulsars, whereas it is 0.2 for long-period ($P \geq 1$ s; usually older) pulsars². Both these factors account for the bias³. In addition, most remnants are visible in the radio or X-ray sky for a short time, typically $\leq 10^4$ yr. An additional complication is that the radio morphology of type Ib and II remnants probably evolves^{6,7} from centre-filled (plerionic) geometry (pulsar powered as in the Crab nebula) to a shell geometry. For these reasons, great importance has been attached³ to the discovery of pulsars in old remnants. Consequently we began a study of pulsars with ages greater than 10^4 yr; here we report the discovery of one such association.

PSR2334+61 was discovered during an extensive survey⁸ for low-luminosity pulsars. Timing observations⁹ showed the pulsar to be young, with a characteristic age $\tau_c = P/2\dot{P} = 4.1 \times 10^4$ yr (see also Table 1). Considerable timing noise is seen, consistent with its young age. The dipole magnetic field strength (B) of the pulsar can be obtained from the usual formula,

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$B = 10^{12} \sqrt{P\dot{P}} = 9.7 \times 10^{12}$ G, significantly above the typical value² of 3×10^{12} G.

The radio nebula G114.3+0.3 (Table 1) was discovered in a 1.4-GHz continuum survey¹⁰ using the Effelsburg 100-m dish. High linear polarization, up to ~60%, established that it was a SNR⁴. An optical H II region, S165 (Sharpless 165; G114.61+0.23) is projected within the radio boundary of G114.3+0.3. There is no diminution in the polarized emission across S165 nor any change in the position angle, demonstrating that G114.3+0.3 is closer to us than S165 (ref. 4). After accounting for S165, the radio spectral index of G114.3+0.3 has been estimated⁴ to be $\alpha \approx -0.3 \pm 0.1$ (flux density at frequency $S_\nu \propto \nu^\alpha$); however, the uncertainty in α , directly estimated from the reported errors in the flux density measurements⁴ is higher, 0.27. By combining the Effelsburg observations with 102.5-MHz observations⁵ obtained with the synthesis telescope of the Lebedev Physical Institute in Pushchino, Russia, we find $\alpha = -0.36 \pm 0.03$, in agreement with that reported in ref. 4. In contrast, the Effelsburg-Pushchino data yield α for the remnants CTB1 and G116.5+1.1 (see Fig. 1) of -0.72 ± 0.03 and -0.85 ± 0.03 , also in excellent agreement with that reported in ref. 4.

From Fig. 1 we see that PSR2334+61 is close to the centre of G114.3+0.3 (see also Table 1). Coincidence in the radial dimension is needed to establish a physical association. The nominal dispersion-measure distance¹¹ to the pulsar is 2.4 ± 0.5 kpc. The distance to G114.3+0.3 can be estimated as it has been argued that this nebula, several other SNRs (CTB1 and G116.5+1.1), H II regions (including S165) and stellar associations are part of a large star-forming complex in the Perseus arm¹². S165 appears to be associated with a molecular cloud whose radial velocity¹³ with respect to the local standard of rest (v_{LSR}) is -33 km s⁻¹. Given the large streaming motions¹⁴ in this region of the Galaxy, kinematic distance estimates such as in ref. 4 are suspect. Distance estimates^{14,15} to about a dozen young stars, open clusters, O associations and H II regions within 10° of this complex place $v_{\text{LSR}} = -30$ km s⁻¹ at a distance of 1.8 ± 0.3 kpc. We conclude that the distance to G114.3+0.3 is also ~2 kpc, consistent with the pulsar distance estimate.

Assuming a simple magnetic dipole model for the pulsar, the true age of the pulsar, and hence that of the remnant, is $\leq \tau_c$. The mean SNR expansion speed is then $\bar{v} = R/\tau_c = 680$ km s⁻¹; here $R = 28$ pc is the radius of the SNR (derived from Table 1). Assuming that the remnant is in the Sedov phase¹⁶, $R = 13 \text{ pc}(E_{51}/n_0)^{1/5}(t/10^4 \text{ yr})^{2/5}$; here E_{51} is the explosion energy in units of 10^{51} erg and is usually set to unity; n_0 is the ambient

particle density in units of cm⁻³. Thus the current shock speed, $v_{\text{sh}} = \dot{R} = (2/5)\bar{v} \sim 270$ km s⁻¹, high enough that the shock is expected to be non-radiative. From the Sedov relation and setting $t = \tau_c$ we find $n_0/E_{51} \approx 2.8$. The swept-up mass is $2 \times 10^3 n_0 M_\odot$, where $M_\odot$ is the solar mass. Furthermore, the location of the pulsar close to the centre of the nebula constrains the pulsar transverse velocity to be ≤ 50 km s⁻¹.

The radio morphology of SNRs containing active pulsars is expected to be centre-filled. Two additional consequences result from having a pulsar continually supply high-energy particles (as opposed to remnants without pulsars, in which such particles are obtained in the strong shock waves at early times and subsequently suffer radiative losses). First, the spectral index is shallow, ~ -0.3 (for example the Crab nebula¹⁷) as opposed to -0.7 for pure shell SNRs (such as CTB1). Second, there is a high degree of linear polarization and simple spatial structure in polarized radiation. In contrast, most shells¹⁸, unless they are young, have complex polarization distribution and fractional polarization of $\geq 5\%$.

The Crab-like spectral index of G114.3+0.3, the centre-filled appearance of the polarized emission, the high fractional linear polarization (typically 30% or greater) and regularity in the polarization vectors (see ref. 4 and in particular Fig. 3c therein) support the view that G114.3+0.3 radio emission is plerionic in origin. If so, the minimum energy in particles and magnetic fields is $5 \times 10^{48} \nu_2^{0.4}$ erg; here, $\nu_2 = \nu_u/100$ where ν_u is the high-frequency cutoff in GHz, and spherical geometry is assumed. The equipartition magnetic field strength is $6\nu_2^{0.2} \mu\text{G}$. Equating the minimum energy to the initial rotation energy of the pulsar (as in ref. 19), $2\pi^2 I P_1^{-2}$, we note that $P_1 < 100 \nu_2^{0.2}$ ms; here I is the pulsar moment of inertia, 10^{45} g cm². As there must be considerable adiabatic and radiative losses, this is a strong constraint. Pulsars born with large period (~ 0.5 s) have been invoked² to explain the paucity of pulsar-SNR associations. It seems that a convincing case is yet to be found.

All-sky X-ray survey data from the Rosat satellite have been searched for emission from G114.3+0.3. At the location of G114.3+0.3, no emission that could be plausibly ascribed to the SNR was detected. Excess X-ray emission has been found along the galactic ridge, $112^\circ \leq l \leq 116^\circ$, $|b| \leq 1^\circ$. The typical surface brightness in the band 0.1–2.4 keV is $(7.3 \pm 1.7) \times 10^{-5}$ count s⁻¹ arcmin⁻². A fit to a Raymond-Smith type model produces a best-fit temperature of 0.5 keV and an interstellar-absorption column density of $N_{\text{H}} = 10^{22}$ cm⁻² for hydrogen. We identify this emission as arising from hot gas in the Perseus star-forming region. The emission is expected to be soft (photon energy $\sim 10^2$ eV). The absence of X-ray emission (3σ upper limit

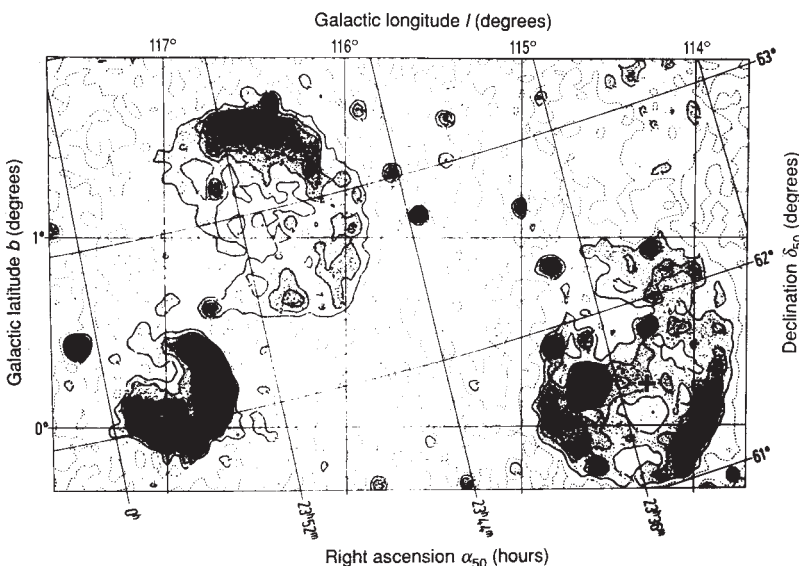


FIG. 1 Total intensity image of the sky at 2.7 GHz of G114.3+0.3 (ref. 2). The position of PSR2334+61 is marked by a cross in the lower right-hand corner. Two other supernova remnants can be seen: CTB1 (due east) and G116.5+1.1 (due northeast). These probably belong to the same star-forming region as G114.3+0.3. The figure is reproduced with permission from W. Reich.

of 1.8×10^{36} erg s⁻¹ for assumed $N_H = 6 \times 10^{21}$ cm⁻²) is explained by invoking possible foreground absorption.

Previous observational and theoretical work has concentrated on young (and hence short-period) pulsars and young remnants. PSR2334+61 is the longest-period pulsar yet found in a SNR. This association and the PSR0656+14/Monogem loop association²⁰ (and perhaps the less firmly established PSR1930+22/G57.1+1.7; ref. 21) are the oldest in which the pulsar is still within the confines of its remnant. If, as we argue above, G114.3+0.3 is pulsar-powered, then its high surface brightness (relative to the Monogem loop and G57.1+1.7) and high polarization are readily explained. Even this small sample shows diversity: G114.3+0.3 is probably a plerion, whereas the

Monogem loop is a shell. G114.3+0.3 also contrasts with CTB1 and G116.5+1.1. The latter two are shell SNRs (judging from their morphology and spectral index) and their location in a star-forming region favours a type Ib/II origin. Such diverse evolution had been suggested earlier and has recently been modelled⁷.

In most other ways, G114.3+0.3 looks like an ordinary SNR. Slow pulsars have a beaming fraction of 0.2, so (statistically speaking) there might be four other SNRs similar to G114.3+0.3 in which the pulsar is undetectable owing to unfavourable beaming. Given the diversity in morphology of old remnants, a careful study of polarization of old remnants may be the best way to infer the existence of pulsars. □

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Rubbery solid electrolytes with dominant cationic transport and high ambient conductivity

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EFFORTS to develop a high-voltage, lightweight rechargeable battery for electrically powered vehicles have focused on batteries based on solid electrolytes, which offer good mechanical strength, easy packaging and high energy densities. An important objective is to identify an electrolyte with the desired combination of mechanical properties, electrical conductivity and stability against powerfully oxidizing and reducing electrodes (lithium is preferred for the anode). Among the most promising materials are rubbery 'salt-in-polymer' electrolytes and highly conducting but brittle superionic glass electrolytes. In the latter category are salts with good lithium-ion conductivity, which are compatible with lithium-anode systems. Here we describe new ionic conductors—'polymer-in-salt' materials—in which lithium salts are mixed with small quantities of the polymers polypropylene oxide and polyethylene oxide. These materials have glass transitions low enough to remain rubbery at room temperature while preserving good lithium-ion conductivities and high electrochemical stability.

The most successful applications so far of the polymer electrolyte principle¹ have involved polymer solvents which have low glass transition temperatures T_g and moderate concentrations of salt^{1–3}. The maximum conductivity is reached at M:O values of ~1:16, where M:O is the mole ratio of salt cation to polymer ether repeat unit. But this maximum conductivity has so far proved inadequate for most practical purposes and it has been necessary to enhance the conductivity by use of plasticizing solvents^{4,5}. Such plasticized 'solid' electrolytes have been proposed⁴ as the most promising materials for battery electrolytes, but they suffer from problems of low cation transport number (and consequent battery polarization) and of slow reaction of

the Li anode with the plasticizer. Alternative methods of improving the conductivity are therefore desirable.

The conductivity of salt-in-polymer solutions is maximal near M:O = 1:16 because the T_g of the solution rises rapidly with salt content in this domain^{1–3}, causing a decrease in ionic mobility which offsets the effect of increasing charge carrier concentration. As the rate of T_g increase is too great to be sustained to much greater salt content⁷, we have explored the range of higher salt concentrations in the belief that T_g will reach a maximum. Then, increases in salt content would not only increase the ion mobility but should simultaneously increase the freedom of small cations like Li⁺ to move independently of their surroundings by making the 'decoupling index' (the ratio of response times to mechanical and electrical stress^{8,9}) more like that of the ionic end member. Ideally, the latter should be a member of the 'superionic glass' family of solid electrolytes^{8,9}. The conductivity could in this case become much higher than that of any salt-in-polymer solution at the same temperature.

Because only small amounts of high-molecular-weight polymer are needed to bestow rubbery properties by means of the entanglement mechanism¹⁰ we can hope to find materials that combine decoupled cation motion with high conductivity and rubbery compliance. For this, a number of conditions must be met. (1) The high salt solution must be capable of cooling without salt crystallization below room temperature while retaining high conductivity. It must also have T_g well below ambient temperature to provide rubbery, as opposed to glassy, compliances when polymer is added. (2) The polymer must be soluble in the salt melt, and a small polymer content must provide rubbery character without reducing the conductivity excessively. (3) The solution with all these properties must be a single-ion (Li⁺) conductor. (4) The electrolyte must have a satisfactory electrochemical stability.

In Fig. 1a we show that lithium salts in appropriate mixtures may indeed be cooled to low temperatures without crystallizing and that certain of these solutions may have T_g well below room temperature. Figure 1b shows T_g for salts added to the polymer, polypropylene oxide, with a molecular weight of 4,000 (PPO 4,000) bearing out our initial conjecture that the T_g of the usual salt-in-polymer systems should pass through a maximum at a

salt content higher than those usually studied. In one case we see that continuous salt-to-polymer solutions are possible.

In Fig. 2a we show conductivity data for a selection of the systems prepared as described in the Fig. 1 legend. These increase in complexity from simple solutions of (1) LiI in lithium acetate (which is a poor conductor at room temperature because of its high T_g) and (2) LiClO₄ in LiClO₃ (which conducts well)

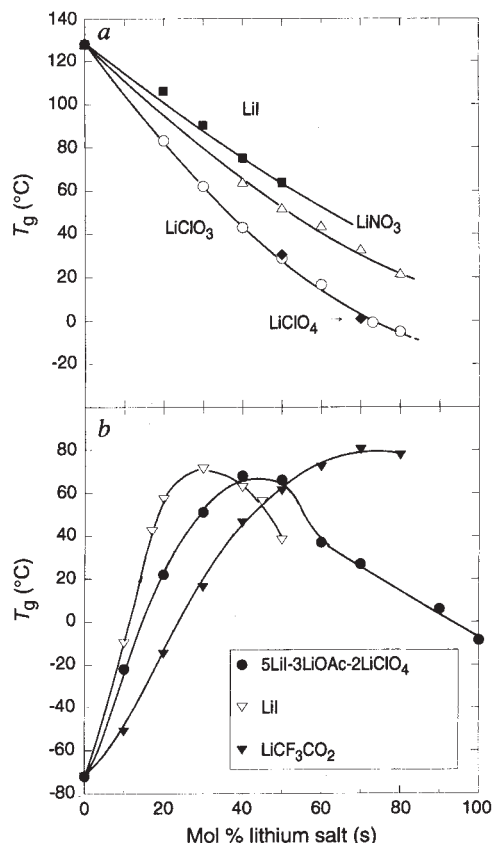
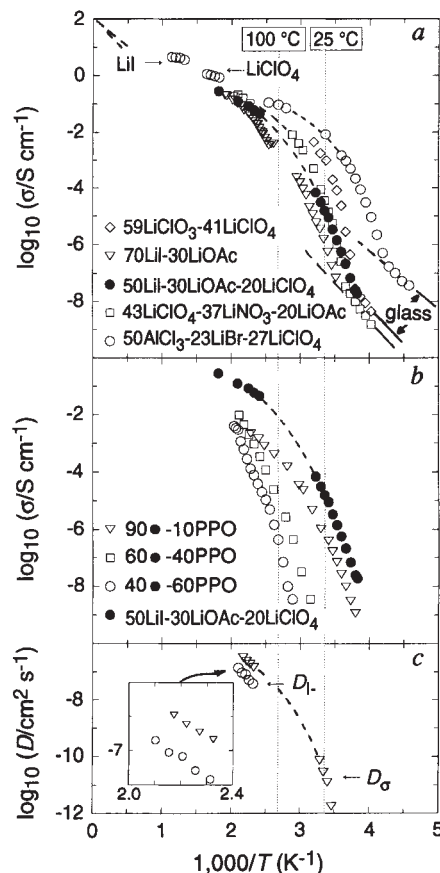


FIG. 2 Conductivity and diffusivity in relation to temperature for glassforming lithium molten salts and polymer-in-salt solutions. *a*, Arrhenius plot of conductivities for different lithium molten salt mixtures and two single salts showing conductivities as high as $10^{-2} \text{ S cm}^{-1}$ at ambient temperature. Note breaks between curvilinear and linear portions of plots due to the freezing of structure into the glassy state below T_g . The pre-exponent for the linear (Arrhenius law) portion in the glassy state is the same as obtained for the common lithium-conducting superionic glasses^{8,9}. From the conductivity at T_g we assess the decoupling indices at T_g , R_g^* (refs 8, 9) for these glasses as $10^{6.3}$ – $10^{7.3}$, values which are much lower than the best possible (10^{14}). For each log unit improvement which can be made in R_g^* there will be about one-third log unit increase in ambient conductivity. *b*, Arrhenius plots for several solutions in the pseudobinary system $x\%$ (50LiI-30LiOAc-20LiClO₄) + $(100-x)\%$ PPO 4,000, showing the effect of polymer additions to the high-conductivity salt melt. The decrease in conductivity is mainly because of the increase of T_g seen in Fig. 1b, which in turn reflects the interaction of the Li^+ ions with the polymer chain ether oxygens. The conductivity in this domain is also adversely affected by the associated increase in coupling between conductivity and viscous modes. *c*, Demonstration that anion diffusivity is too small to account for the observed conductivity of our melts. Solid data points are for iodide ion self-diffusivity in 25 LiOAc + 75 (46.5 LiNO₃-53.5 LiClO₄) measured in the high-temperature range by chronopotentiometry and chronoamperometry; these electrochemical techniques give results in agreement to $\pm 10\%$. Triangles show how large the average diffusion coefficient D_{av} of monovalent ions in the melt must be to account for the observed conductivity (hereafter called D_σ); this is calculated using the Nernst-Einstein equation, $\sigma = (F^2/VRT)(D^+ + D^-) = (2F^2/VRT)D_{av}$, where F is the Faraday, V the molar volume, and D^+ and D^- are the individual monovalent ion self-diffusivities. I^- is the smallest anion in the melt; since Fig. 2c shows that its diffusivity accounts for $\leq 20\%$ of D_σ , even at high temperatures where cation/matrix decoupling is greatly reduced^{8,9}, the conductivity at ambient temperatures must be dominated by the cation Li^+ .

to a three-salt mixture containing AlCl_3 which has the extraordinarily high conductivity of $1.0 \times 10^{-2} \text{ cm}^{-1}$ at ambient temperature. The latter cannot be used in a rechargeable battery with lithium anodes, but it indicates the conductivity which in principle can be reached with lithium salts having the right combination of size and charge. The 100 °C conductivities of many salt combinations exceed those of all simple lithium

FIG. 1 Glass transition temperatures for salt and salt plus polymer systems studied here *a*, Solutions of various lithium salts in the common-component salt, lithium acetate (LiOAc). *b*, Solutions of salts or salt mixtures, as marked on diagram, in the common component polypropylene oxide of molecular weight 4,000 (PPO 4,000). Note the T_g maxima in two cases: these serve to separate the 'salt-in-polymer' from 'polymer-in-salt' domains. *a* suggests combinations of salts which may be used to give crystallization-resistant melts of low T_g . The simplest case would be LiClO₃ + LiClO₄, which indeed proves to be glassforming with $T_g = -10^\circ \text{C}$. *b* shows that one of the glass-forming mixtures suggested by *a* can undergo a continuous transformation from pure polymer to polymer-free ionic liquid. To obtain the solutions for these measurements, reagent-grade salts were dried under vacuum at 100–150 °C, mixed and melted in stoppered vials. In the case of chloroaluminate, dry box preparations were used. Salt-in-polymer solutions were prepared by direct dissolution of salts in PPO 4,000 (from Aldrich). For the preparation of polymer-in-salt solutions or any solutions with high-molecular weight polymer (for example PPO 10⁶ or polyethylene oxide of molecular weight 10⁵ (PEO 10⁵) it was necessary to use a molecular solvent, either acetonitrile or acetone. The solvent then had to be removed by boiling followed by prolonged vacuum evaporation at increasing temperature up to 120 °C to remove the last traces. Removal of solvent was confirmed by weighing. Glass transition temperatures T_g were measured using a Perkin Elmer DSC-4 with scans at 20 K min⁻¹. Conductivities were determined using dip-type cells with platinum wire or parallel plate electrodes.



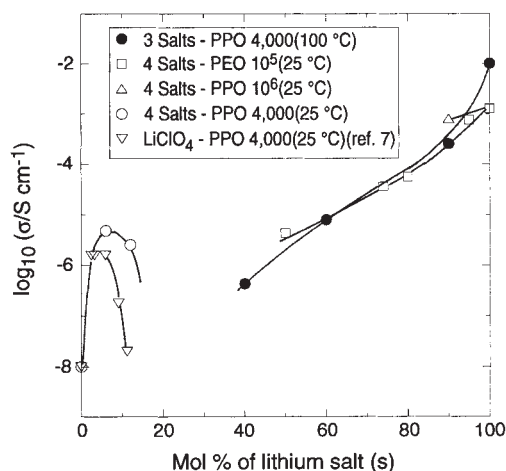


FIG. 3 Composition dependence, at 25 and 100 °C, of conductivity in systems of polymer plus lithium salt(s). The conductivity, which initially reaches a maximum at ~6–8% salts, rises again at higher salt contents and reaches values far above that of the initial maximum. The 25 and 100 °C conductivities of the rubber made up of 90% (four salts) + 10% (PPO 10⁶) may be compared with those of other good solid and liquid lithium electrolytes; for example, propylene carbonate-plasticized polymers^{4,5} (5×10^{-3} and 1.1×10^{-2} (extrapolated) S cm⁻¹ at 25 °C and 100 °C respectively), superionic glass, 45LiI–55LiP₂S₇ (1.0×10^{-3} and 1.0×10^{-2} S cm⁻¹; ref. 14), organic non-aqueous electrolyte solution LiN(CF₃SO₂)₂ in 1:1 propylene carbonate-dimethyl ether¹⁵ (1.2×10^{-2} and 2.9×10^{-2} (extrap.) S cm⁻¹) and inorganic non-aqueous electrolyte solution, 1.73M LiAlCl₄ in SO₂Cl₂,¹⁶ (2.0×10^2 S cm⁻¹ at 25 °C).

electrolytes except for aqueous solutions. Figure 2a also shows that liquids based on combinations of the lithium salts used in Fig. 1a can be studied over the many hours needed to acquire the conductivity data for an Arrhenius plot without crystallization occurring.

To see the effect of dissolved polymer on the conductivity, we dissolved varying amounts of polymer (PPO 4,000) in a three-salt melt (Fig. 2b). The lower-molecular-weight polymer was used for ease of sample preparation. Separate experiments on solutions with high-molecular-weight polymers, which are much more difficult to introduce into the conductivity cell because of their strong rubbery character, indicate that the conductivity behaviour depends little on the polymer molecular weight. Roughly 10–20% repeat units of high-molecular-weight polyether (depending on the actual molecular weight) is needed to give the salt melt true elastomeric properties. Figure 2b shows that, for the polymers considered here, we must accept at least one order of magnitude lower conductivity to obtain an ionic rubber for solid electrolyte applications. The acetate-based system of Fig. 2b performs poorly, but with an acetate-free salt melt (obtained by substituting LiClO₃ for Li acetate) we can obtain a conductivity of $\sim 10^{-4}$ S cm⁻¹ at ambient temperature, even with 20% polymer repeat units. Isotherms for two systems are shown in Fig. 3. This figure shows how replacement of one anion by another can be as effective as raising the temperature by 75 K, and indicates a possible avenue to improvement. This plot shows how far the conductivities in the polymer-in-salt domain can surpass the maximum value obtainable in the 'normal' salt-in-polymer domain.

As yet, we have not evaluated the rubbery moduli and compliances. For the present, we consider the material to have elastomeric properties if fibres, pulled from the hot sticky melt and cooled, behave like rubber bands with strongly elastic properties. The present type of system with more than ~10% of PPO 10⁶ or ~15% PEO 10⁵ produces strong rubbers which adhere, like heavy-duty rubber cements, to metal surfaces. This is a desirable

feature for a solid electrolyte.

We now turn to the third of the above conditions: that the conductivity of the materials be dominated by the Li⁺ cations, as in the Li⁺-based fast-ion glass systems. Although this must hold for the glassy state of our materials, it is natural to question it for the temperature range above T_g , because all particles present must have some mobility in the liquid state. The important question, however, is the relative mobility of Li⁺ compared with other species.

There are ways to establish that Li⁺ does dominate the conductivity without measuring the mobilities of all species or attempting to measure a transport number (which is a contentious concept in molten salts¹¹). One is by deduction, using the variation of decoupling index R_T with temperature above T_g . This has been assessed for a classical alkali borate system: Fig. 1 in ref. 8 suggests that, at $T/T_g = 1.2$ (characteristic of our systems at 25–50 °C), the R_T should be $\sim 10^2$, so the transport number should be ~ 0.99 (or 0.9 for our less decoupled system). A second method (see Fig. 2) is to measure the diffusivity of a typical anion, I⁻, by an electrochemical technique, and show that it is too small to explain the conductivity. A third¹² is to observe that the anomalous relation between ⁷Li NMR spin-lattice correlation times and conductivity relaxation times seen in superionic glasses^{9,13} is also found in our liquid electrolytes. The anomaly disappears as the salt-in-polymer domain is approached¹².

Finally we address the fourth condition, electrochemical stability. We have observed decomposition voltages (from current-voltage plots) of up to 4.8 V in systems (not described here) with ambient conductivity of 10^{-4} S cm⁻¹ in which we are confident no traces of water remain. In the LiClO₃-containing rubber (4-salts) which shows high conductivity in Fig. 3, the current-voltage plot indicated a lower electrochemical 'window', only ~2.5 V, but LiClO₃ is notoriously hygroscopic and the window was probably determined by trace water/nitrate reduction. The electrochemical aspects of our work are at present the least well developed. □

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Anomalous solubility behaviour of C₆₀

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SOLUBILITIES of solids in liquids exhibit a temperature dependence that is correlated with the energetics of dissolution. For organic solutes in organic solvents the common experience is that solubility increases on warming (that is, dissolution is normally endothermic), forming the basis for purification by crystallization by slow cooling of hot solutions¹. Interactions between inorganic compounds and water are often more energetic, and may lead to complicated temperature-dependent solubilities, often associated with the formation of hydrated solid phases². We have investigated the temperature-dependent solubility of C₆₀ in hexane, toluene and CS₂. We observe a solubility maximum near room temperature (around 280 K) for all three solvents. Although the solubility of C₆₀ in these three solvents differs by several orders of magnitude, the temperature dependence of the relative solubilities is much the same in each case. We conclude that dissolution is endothermic below room temperature and exothermic above. We interpret this change as being due to a phase change in solid C₆₀, presumably the phase change observed previously in the absence of a solvent³⁻⁵, modified by solvent wetting. A solubility maximum (or minimum) for organic compounds in non-electrolytes is highly unusual, and may be unprecedented. The effect may have consequences for solvent-extraction techniques of fullerene purification.

Our aim was to find more efficient procedures for extracting and separating fullerenes, prompted in part by the observation⁶ that chromatographic retention times for C₆₀ and C₇₀, on a Regis (R)-N-(3,5-dinitrobenzoyl)-phenylglycine column using 10% dichloromethane in hexane as the mobile phase increased when the temperature was raised from ambient to 90 °C.

The apparatus used to determine the solubilities consists of a pyrex U-tube with a fine sintered-glass frit (4 µm) in one of the arms. The apparatus is immersed in an insulated glass bath. For our initial experiments we placed constant-temperature slushes in the bath; for subsequent experiments we used refriger-

ated fluids circulated through a copper tube in the bath. Excess C₆₀ and solvent are placed in the arm without the frit to make saturated solutions. After stirring in the dark for a minimum of 12 hours, the saturated solution is forced through the frit by pressurizing the arm with argon. The solution is forced in and out of the filter arm four or five times to condition the filter in case it absorbs some C₆₀. An aliquot of 1–2 ml is removed with a pipette from the filter arm for analysis by high-performance liquid chromatography (HPLC) either with further dilution (as in the case of toluene and CS₂) or without (hexane).

To keep the solution at constant temperature, the bath is stirred, and the U-tube is positioned such that about 5 cm³ of bath liquid is above the frit. Calibrated thermometers are placed in the bath and the arm containing C₆₀, and always read (to within 0.2 °C) the same temperature. The highest temperatures achieved for each solvent were the atmospheric-pressure boiling points: 46 °C for CS₂, 68 °C for hexanes, 110 °C for toluene. A reflux condenser was fitted to the U-tube for the boiling solutions.

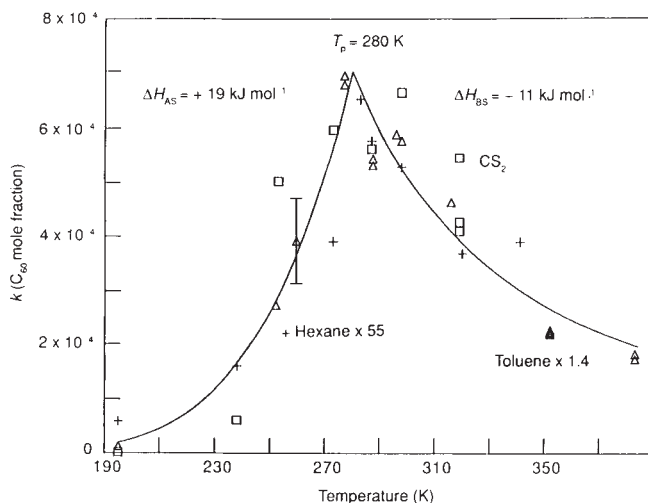
The solubility of C₆₀ at room temperature in hexane, toluene and CS₂ was determined to be 0.04, 2.8 and 7.9 mg ml⁻¹ respectively. Expressed in mole fractions, the room temperature solubility is 0.073×10^{-4} in hexane, 4.1×10^{-4} in toluene and 6.6×10^{-4} in CS₂. The temperature dependence of the solubilities (mole fractions) is shown in Fig. 1. From replicate determinations, we estimate the reported solubilities (*k*) to be within 20% of the true values. In all three solvents, the solubility of C₆₀ is minimal at -78 °C. Warming increases the solubility of all three solvents, albeit to different extents. The trend continues up to ~10 °C, above which it is roughly flat till 25 °C and then decreases by a factor of about 2–3 from 25 °C to the normal boiling point of each solvent.

Room-temperature solubilities were also independently determined by filtration with a 0.45-µm polytetrafluoroethylene (PTFE) filter (Gelman Acrodisc)⁷; the measured solubilities were identical to those determined with the U-tube apparatus used here. This agreement confirms that the glass filter (~4-µm) was fine enough to preclude small particles from contributing to the measured solubilities.

Several additional tests were done to verify the unusual solubility behaviour of C₆₀ in each solvent and to rule out experimental artefacts. A room-temperature saturated toluene solution was filtered with a 0.45-µm PTFE filter, and subsequently placed

FIG. 1 Temperature dependent solubility of C₆₀ in hexane (+, ×55), toluene (Δ, ×1.4), and CS₂ (□). The phase-transition model is represented by the solid line, with a transition temperature of 280 K and enthalpy change of 30 kJ mol⁻¹.

METHODS. Pure C₆₀ (99.5%) was obtained by extraction from Krätschmer-Huffman soot followed by column chromatography on neutral alumina with hexane eluent, and drying for 24 hours in a vacuum oven at 100 °C. A separate U-tube was used for each of the three solvents: hexanes (Mallinckrodt HPLC grade, 98% *n*-hexane, boiling range 68.3–68.9 °C), toluene (Mallinckrodt HPLC grade, 99.9%, boiling range 110.5–110.6 °C), and CS₂ (Baker analysed reagent grade, 99% by GC, boiling point 46.3 °C). HPLC analyses were conducted using a Waters System 6000 Chromatography equipped with a DNAP column (9 mm × 250 mm; E. S. Industries), an ultraviolet detector set at 340 nm, and an integrator. A solution of toluene (20% by volume) in hexane was used as the eluant. Under these conditions, better than baseline separation between C₆₀, C₇₀, and higher fullerenes is achieved to permit quantitative analysis. Detector response for C₆₀ was calibrated using several standard solutions in the range of 0.01 to 0.20 mg ml⁻¹. A representative error bar is shown for the solubility of C₆₀ in toluene at 260 K. Saturated solutions of C₆₀ in toluene and in CS₂, which are generally much more concentrated than the highest concentration used for calibration were diluted (typically, 20- to 100-fold) before analysis. In the case of hexane, an aliquot of the solution at temperature was removed and later, when the solution was at room temperature, 20 µl were injected into the chromatograph. Corrections for density changes are therefore warranted only in the case of hexane. The concentrations reported here have been corrected using 0.095% per °C as the volumetric coefficient of expansion for hexane⁹.



in a bath at 80 °C. A solid precipitated out, and the intensity of the saturated solution in the vial had visibly diminished: we could see through the vial quite easily. The saturated solution with solid precipitate was then cooled to room temperature, and placed in an ultrasound bath for ~20 s. All of the solid precipitate returned to solution. The cycle was repeated several times, with the same results.

A second test to ensure equilibration was to determine the solubility at room temperature over different time intervals of stirring: 12 hours, 2 days, 7 days. The solubilities in hexane, for each time interval, were in agreement within 5% as determined with calibrated HPLC. Because the solubility is close to a maximum at room temperature, we believe that equilibration should have occurred on the 12-hour timescale used at all other temperatures; we have approached both higher and lower temperatures starting with solutions equilibrated at room temperature.

The unusual temperature dependence is likely to be caused by the changes in the solid phase. We arrived at this hypothesis as a result of recognizing the nearly identical temperature dependence of k in three different solvents, although the absolute value of k in these solvents varies by about two orders of magnitude. Crystalline C_{60} has a phase transition at about 260 K, and the temperature width of the transition is influenced by the degree of crystallinity present. Differential scanning calorimetry has been used to obtain ΔH for this first-order phase transition. Two experimental values for ΔH are 6.6 and 7.0 kJ mol⁻¹ (refs 4, 5). First-order phase transitions in the solid phase cause a change in slope of $k(T)$ at T_p , the phase transition temperature. Thus, one might think that the observed $k(T)$ can be rationalized if $H_A < H_B < H_s$, where H_A , H_B and H_s are respectively the enthalpies of the low-temperature phase (A), the high-temperature phase (B), and the solution (s). We find, however, that the first-order phase transition of pure C_{60} cannot by itself explain the observed solubility behaviour.

Under the assumption that there is only a narrow region of coexistence, we can model the observed solubilities by equations of the form

$$k(T) = \exp(\Delta S_{AS}/R - \Delta H_{AS}/RT) \quad \text{for } T \leq T_p \\ = \exp(\Delta S_{BS}/R - \Delta H_{BS}/RT) \quad \text{for } T \geq T_p$$

These equations were used to fit the data shown in Fig. 1, the result being shown by the solid line. The parameters derived are sensitive to the fact that the model requires a sharp maximum in contrast to the observed broad maximum. Reasonable fits can be obtained for $T_p = 280 \pm 10$ K, $\Delta H_{AS} = 19 \pm 3$ kJ mol⁻¹, $\Delta H_{BS} = -11 \pm 2$ kJ mol⁻¹, and $\Delta H_{AB} = 30 \pm 3$ kJ mol⁻¹. The transition temperature is higher, and the transition enthalpy about five times larger, than observed previously in pure C_{60} (refs 3–5). The ΔH for the first-order phase transition of pure C_{60} (~7 kJ mol⁻¹) therefore cannot explain the data. But it is reasonable to conclude from our simple model that a phase change is occurring. A possible interpretation for the substantially larger enthalpy change for the phase transition is that the properties of the solid phase have been modified by solvent wetting. Although it is known empirically from previous studies³ that solvents adhere strongly to C_{60} , leading to difficulty in drying, it is surprising both that the effect would be as large as observed and that hexane, toluene and CS₂ would all show the same influence.

The unusual temperature dependence of the solubility of C_{60} is probably playing a role in a recently reported C_{60} purification procedure⁸. Toluene soxhlet extraction of a primary fullerene soot removes a roughly 80:20 mix of C_{60} : C_{70} . Control of the ratio of primary fullerene soot mass to volume of toluene, and of the extraction time, allows collection of crystals which are 98% pure C_{60} . The collection pot is held at the boiling point of toluene (110.6 °C) during the 10-hour soxhlet extraction⁸. A likely explanation is that the temperature dependence of the

solubility of C_{60} and C_{70} differ, with C_{60} having lower solubility in the boiling toluene.

This simple model explains only the salient features and does not fit the data perfectly. To understand the temperature dependence fully, we need, for example, to characterize the solid phases A and B, and to measure the heat of solution as a function of temperature. □

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Vast Neogene laminated diatom mat deposits from the eastern equatorial Pacific Ocean

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THE eastern equatorial Pacific upwelling region is responsible for up to 50% of global 'new' production¹ and is regarded as a barometer of ocean change. Here we report evidence, from several sediment cores in the region, for repeated episodes of increased equatorial primary production between 15 and 4.4 million years ago, on a scale that is undocumented in the modern ocean. Mats of the diatom *Thalassiothrix* were rapidly deposited as successive laminations at rates exceeding 10 cm per thousand years; mat deposits can be correlated for distances of more than 2,000 km. It is surprising that the laminations were preserved at all: conventional models suggest that bioturbation caused by the benthic sediment community will disrupt such laminations unless there is insufficient oxygen in the water to support the biological activity. In this case, however, the strength of the mats and the scale of their deposition may have overwhelmed the benthos, so that the laminations were preserved by physical means. These remarkable deposits provide a unique window into the Neogene tropical ocean-climate system, and should enable quantification of ancient deep-sea fluxes and the study of short-term (sub-Milankovitch) variability in the ocean.

As a major zone of primary production, the eastern equatorial Pacific continues to be the focus of studies of modern and geological oceanic and climatic processes^{1–5}. Leg 138 of the Ocean Drilling Program (ODP) has provided a high resolution record of the last 15 million years depositional history of the low-latitude Pacific between 90° and 110° W. Intervals of highest biosiliceous phytoplankton abundance are recorded by near monospecific assemblages of the pennate diatom *Thalassiothrix* present in laminated diatom ooze (LDO) at ODP Sites 844, 847, 849, 850, and 851 (Figs 1 and 2). Re-examination of core material from Deep Sea Drilling Project Sites 572–574 confirms the occurrence of similar laminated diatom ooze at least as far as 133° W. Laminated sediment occurs intermittently in the above sites from 15 to 4.4 million years ago (Myr BP) but is concentrated at about 15, 13–12, 10.5–9.5, 6.3–6.1, and 4.4 Myr with a

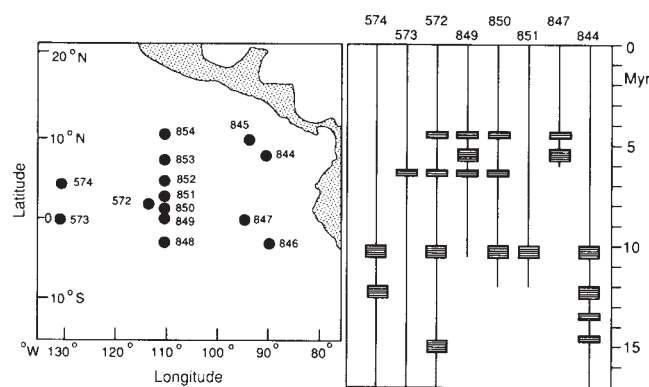


FIG. 1 Location map (left) and summary of occurrences of the major intervals of laminated diatom ooze (right) in ODP Leg 138 sites and DSDP sites 572–574. These intervals comprise alternations of laminated and partly bioturbated laminated diatom ooze with nannofossil ooze or diatom nannofossil ooze.

more geographically restricted interval at 5.8–5.1 Myr. No laminated sediments occur after 4.4 Myr. Combining these data sets with plate motion backtrack data shows that all laminated intervals were deposited when the sites lay within 2° of the Equator.

Preliminary micropalaeontological studies of laminated intervals show that *Thalassiothrix longissima* is dominant although minor occurrences of other diatoms of the *Thalassiothrix* group have been noted. The presence of *Thalassiothrix* in Neogene sediments from the eastern equatorial Pacific has been widely used as an indicator of significant upwelling and high primary production^{6,7}. *Thalassiothrix longissima* (characterized by long straight to twisted cells, only 1.5–5 µm wide but up to 4 mm long) is described as a cosmopolitan species with a distribution ranging from the Southern Ocean to the North Pacific, North Atlantic and the Norwegian Sea^{8–10}. This apparent widespread distribution, however, may result from differences in species definition and the grouping of various forms of *Thalassiothrix* sp. (such as *T. antarctica*, *T. acuta*, or *T. heteromorpha*) into *T. longissima*¹⁰. In the modern ocean, *Thalassiothrix* ranges in abundance up to 10³–10⁶ cells per litre and occurs as single cells, colonies, or arranged in bundles and large tangled masses or mats^{11–13}. There is no information on the sinking characteristics of *Thalassiothrix* mats but new data from sediment trap studies have highlighted the significance of mass sinking of mats of rhizosolenid diatoms as agents of rapid flux to the sea floor¹⁴. The recently reported masses of *Rhizosolenia* associated with a major front in the equatorial Pacific in the fall of 1992 (ref. 15) may provide a modern analogue for the origins of the *Thalassiothrix* mat deposits.

The sub-millimetre (typically 20–300 µm thick) lamination is principally due to an interlocking mesh of *Thalassiothrix* (Fig. 3a). The composition of individual laminations, determined by electron microscopy, varies from near monospecific assemblages of intact specimens to mixed assemblages of *Thalassiothrix* with calcareous nannofossils and/or centric diatoms. Other laminations comprise fragmented and pelletized diatoms or nannofossil-diatom ooze. Foraminifers occur abundantly in the more calcareous horizons and rarely (but persistently) within the *Thalassiothrix* laminations (Fig. 3b). Individual laminations are defined by differences in microfossil assemblage (Fig. 3b) and/or by bed-parallel discontinuities in the *Thalassiothrix* meshwork. Some individual laminations may be traced across the cross-section of the core implying an original mat area of more than 30 cm². We interpret the bed-parallel discontinuities in the *Thalassiothrix* meshwork as representing the contact between successively deposited mats of diatoms, and the laminations (defined by differences in microfossil assemblage) as rep-

resenting the deposits of different episodes of mat production.

Laminated sediment is typically concentrated in packets 10–20 m thick. Within these packets, there are commonly three scales of alternation between LDO and the regionally dominant sediment type of foraminifer-nannofossil ooze: at the sub-millimetre, the millimetre to centimetre, and the decimetre scale. The sub-millimetre laminations are discussed above. The mm- to cm-scale alternation is between paler (nannofossil-bearing) diatom ooze and purer diatom ooze (Fig. 2). On a decimetre scale there is an alternation between beds of unlaminated nannofossil ooze and subordinate quantities of laminated diatom ooze and/or partly bioturbated diatom ooze, with fragmented mats. Exceptionally, laminated beds over 0.5 m thick are present. Many LDO beds (for example, 4.4 Myr) have a very sharp base marking an abrupt transition from nannofossil ooze to diatom ooze. This represents the very abrupt onset of a period of sustained high productivity and diatom mat flux. Within laminated episodes, individual beds may be correlated over great distances. For example, the distinctive 60 cm bed of laminated diatom ooze which occurs at the base of the 4.4 Myr interval is present at sites 847, 849, 850 and 572 and so may be correlated over a longitudinal extent of at least 2,000 km. (Correlation is based on biostratigraphy and continuous Gamma Ray Attenuation Porosity Evaluator (GRAPE) data^{5,16}, as well as lithostratigraphy). The coincidence of the laminated intervals with abrupt changes in sediment density, and hence seismic impedance contrast, will provide the means to correlate depositional events over great distances from seismic records¹⁷ in a critical area of the ocean basins.

FIG. 2 Core photo showing a rare, saw-cut surface of the laminated diatom ooze. A millimetre to centimetre-scale alternation between darker (green) pure diatom ooze and paler (off-white) mixed diatom and calcareous nannofossil ooze is the most obvious feature (core 851B-29X-5, 84–100 cm). Wire core splitting caused considerable deformation of laminated intervals making recognition of laminations difficult but producing a characteristic roughened surface. Obvious laminations were only revealed when a saw was used for core splitting. This happened only for the deeper intervals and also had the effect of nearly destroying the core.



The most surprising feature of the laminated sediments of the eastern equatorial Pacific is that they are preserved at all in such widespread oxygenated deep sea environments. The preservation of laminated biogenic sediments is currently understood to be the result of inhibition of benthos by low concentrations of dissolved oxygen either in anoxic silled basins (such as the Black Sea¹⁸ or California Borderland Basins)¹⁹, or beneath zones of strong upwelling, where an oxygen minimum layer intersects the shelf or slope (such as off Peru²⁰ or within the Gulf of California)²¹. Studies of benthic foraminifera (sensitive indicators of oxygenation) through the laminated intervals²² show no evidence for any increase in species characteristic of a low oxygen environment, but rather a decrease in tapered cylindrical and infaunal forms attesting to the impenetrability of the meshwork. Where limited bioturbation of the mat deposits is observed, burrows parallel to bedding (and mats) occur but vertical burrows penetrating the mats are absent. Such is the tensile strength of individual laminations that some are seen to have been partially pulled out of the core during piston coring or by the wire during core splitting. It is therefore possible that the common absence of bioturbation in laminated intervals is best explained by the high tensile strength and impenetrability of the diatom meshwork in the *Thalassiothrix* laminations, which physically suppress benthic activity.

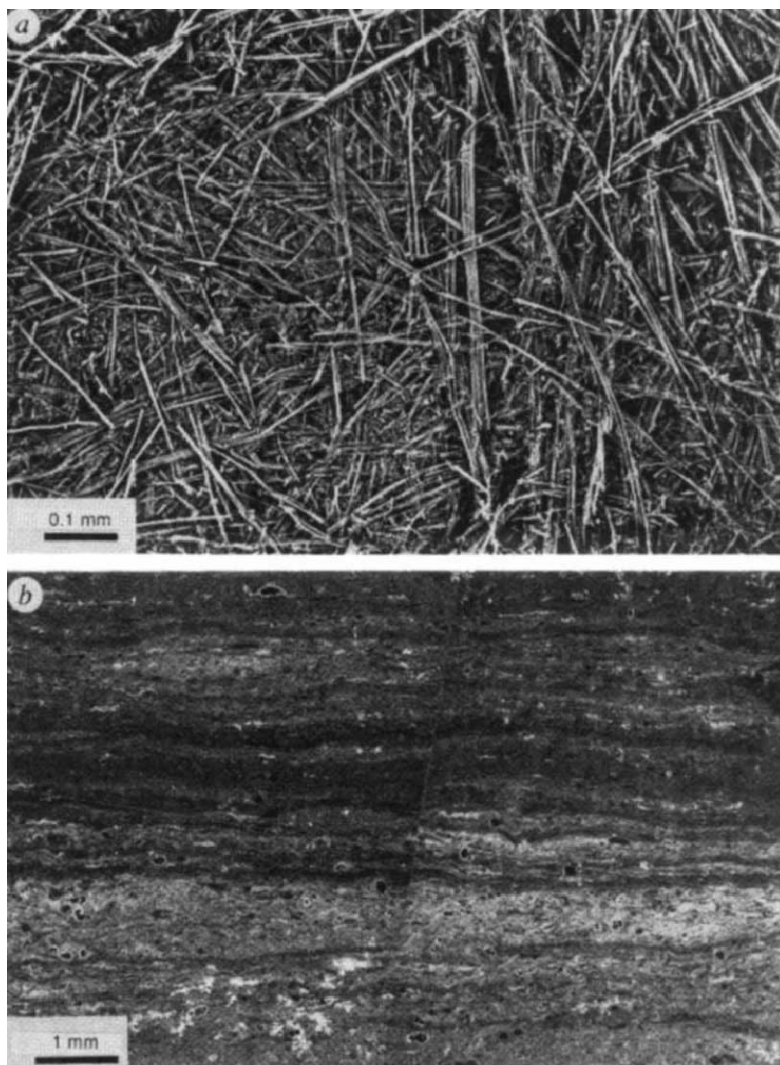
The physical suppression of bioturbation by diatom mats as the mechanism for preservation of lamination in the *Thalassiothrix* oozes of the eastern equatorial Pacific has major implications for the origins of other enigmatic, near-monospecific deep sea

diatom oozes of Neogene and Quaternary age^{23–26}, some of which are macroscopically laminated and whose origins have previously been ascribed to reduced oxygen conditions^{25,26}.

Sedimentation rates for intervals containing a high proportion of laminated sediment are commonly around 10 cm per thousand years (cm kyr^{-1}) (ref. 5). Within these intervals, the LDO is interbedded with foraminifer nannofossil ooze, a lithology characterized by sedimentation rates of the order of 2–4 cm kyr^{-1} . Thus, the 10 cm kyr^{-1} rate for the LDO may be regarded as a minimum. Calculations using this minimum sedimentation rate, and taking an average lamina thickness of 100 micrometres, suggests at least annual deposition of mats, although more than one mat layer may have been deposited from a single mat forming event (concentrations of up to 4.4 m^{-3} have been reported for *Rhizosolenia* mats¹³).

Quaternary variation in carbonate and silica abundance in deep sea sediments is thought to be largely determined by the balance between the surface production of calcium carbonate and its dissolution in the undersaturated deep waters²⁷. There is, however, no evidence to support preferential dissolution of carbonate in the *Thalassiothrix* laminations (Fig. 3b, and above). The *Thalassiothrix* mat deposits, which are the direct result of surface processes, are therefore clear evidence that surface processes, and not dissolution at depth, controlled many of the major changes in the relative abundance of carbonate and silica during the Neogene at least in the equatorial Pacific. Furthermore, the formation of mats of diatoms and their subsequent sinking distorts the normal carbonate-silica cyclicity observed.

FIG. 3 Scanning electron microscope images of diatom mat deposits. *a*, A diatom mat—a secondary electron (topographic) image showing the surface of a peeled *T. longissima* lamina (mat). The individual frustules in this example are between 3 and 5 μm wide but up to 3 mm long. In addition to the general interlocking meshwork arrangement of the frustules, sheaf-like bundles are present. Both the bundles and interlocking meshwork are characteristic features of present day net hauls of *T. longissima* mats (compare Fig. 15a and b in Ref. 11). The diatom mat forms an impenetrable meshwork of great tensile strength which physically suppresses benthic activity. *b*, A back scattered electron image of a polished section (cut perpendicular to bedding) of resin-impregnated sediment showing fine detail of laminations defined by differences in composition. This image can be most simply regarded as a porosity map, since the impregnating resin has a relatively low atomic number and shows up black. The darkest (high porosity) laminations are the monospecific concentrations of *T. longissima* (the diatom mats). The paler (lowest porosity) laminations comprise mainly coccoliths with a mixture of varied diatom flora and foraminifers. The small (0.1–0.3 mm) black objects, commonly with a white outline, are planktonic and benthic foraminifers. These are most abundant in the paler, carbonate rich zones but are also present in the darker diatom mat laminations.



Clearly, the mats are deposited much more rapidly than the surrounding nannofossil ooze so that the assumptions of uniform sedimentation rates between age 'picks', upon which most palaeoceanographic techniques rely, are invalid. Indeed, the signal from the mat flux episodes may have to be filtered out to permit conventional Milankovitch band analysis. For example, the GRAPE record (giving carbonate–opal variation¹⁶) generally shows excellent correlation between equatorial sites. However, this correlation breaks down when comparing intervals where one site has significant LDO and another has none.

At least three sub-Milankovitch band periodicities are present in laminated intervals: individual (sub-millimetre) laminations must represent the fall out from mat-forming episodes and, by consideration of sedimentation rates (above), record events of the order of annual average frequency. The millimetre to centimetre scale alternations, which record periods of more or less intense upwelling, represent time scales of several years to several tens of years and may represent a response to some internal oscillations of the equatorial Pacific such as El Niño. The decimetre-scale interbedding between LDO and nannofossil ooze represents timescales from hundreds to a few thousand years. In addition a coarser time scale of LDO occurrence exists (Fig. 1) which may be related to intervals of major global ocean–atmosphere reorganization.

The period of deposition of the laminated ooze (from at least 15 to 4.4 Myr) coincides with episodes of major cooling and ocean reorganization. Although at present, age controls are tentative and further work will be necessary to substantiate correlations, the following points are worthy of note. The 10.5–9.5 Myr episode is penecontemporaneous with a shift in the main locus of silica production from the Atlantic to the Pacific^{28–30}. The stopping of laminated sediment deposition after the 4.4 Myr event (Fig. 1) coincides with a shift in silica production from the equatorial Pacific to the Antarctic circumpolar ocean^{31,32}. The absence of the laminated intervals in sediments younger than 4.4 Myr further suggests that nutrients, in particular silica, were not supplied in sufficient quantities to fuel these bursts of export production in the eastern equatorial Pacific after this date.

This new evidence for rapid sedimentation of diatom mats throughout large areas of the eastern equatorial Pacific suggests major draw down of carbon, silica and other nutrients. Assessment of the extent to which the widespread, geologically correlated occurrences of mat deposits (Fig. 1) represent synchronous deposition will depend on continuing refinement of Leg 138 time scales but may have major implications for chemical budgets and the rates of biogeochemical cycling. □

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Generation of sodium-rich magmas from newly underplated basaltic crust

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SODIUM-RICH rocks of trondhjemite–tonalite–dacite (TTD) or –granodiorite (TTG) suites form much of Precambrian continental crust¹. They are thought to have formed by partial melting of subducted oceanic crust^{2,3}—a process that would have been much more widespread early in Earth history than at present, owing to the higher thermal gradients prevailing at that time⁴. Phanerozoic TTD suites do exist, however, and seem also to relate to subduction zones⁵. Defant and Drummond⁶ proposed that these suites form where young (<25 Myr), hot oceanic lithosphere is subducted and melts, thus locally simulating the conditions that led to widespread crustal growth in the Archaean. Here we describe plutonic and volcanic rocks from the Cordillera Blanca complex in Peru, which have characteristics of the high-Al TTD suite but which were produced above a subduction zone containing a 60-Myr-old slab. We present evidence that the complex formed by partial melting of newly underplated basaltic crust, and argue that this mechanism should be considered more generally as an additional way of generating sodium-rich arc magmas.

Where subducting lithosphere is sufficiently hot, it can melt before it dehydrates, leaving a garnet–hornblende residue that produces the low Yb contents and high La/Yb ratios characteristic of high-Al TTD suites. Slabs being subducted today at the rims of large ocean basins are typically >60 Myr old, and are accordingly cold enough to dehydrate before melting⁷. Magma formation is thus confined to the hydrated mantle wedge above the subducting slab, where residues of mainly olivine and pyroxene give rise to calc-alkaline magmas with higher Yb contents and lower La/Yb ratios than in the high-Al TTD suites. The change from high-Al TTD to basalt–andesite–dacite–rhyolite generation is thus symptomatic of the secular cooling of the Earth².

The Cordillera Blanca complex, which lies inboard of the Coastal Batholith, marks the culmination of Andean magmatism in northwest Peru (Fig. 1). It is made up of coeval ignimbrites (K/Ar date 4.65–6.7 Myr, from five dates over a 50-km section^{8,9}) and a large batholith containing a variety of rock types but dominated by granite (SiO₂ > 70%) with an ⁴⁰Ar/³⁶Ar age of 5.18 ± 0.05 Myr^{10–12}. The ignimbrites were collected in a strike-slip rift valley (100 × 15 km) related to the Cordillera Blanca fault system, an important crustal structure which marks the western boundary of the batholith (Fig. 1) and is thought to extend to depth to the source region of the complex^{12,13}. The batholith formed during transtensional movements on this strike-slip structure¹⁴. The complex overlies the newly thickened, Miocene continental keel of the Andes, here ~50–60 km thick¹⁵.

The compositions of the ignimbrites and granites are similar to Archaean and Proterozoic trondhjemites (Table 1), with high Sr and Na₂O, and low Y and heavy rare-earth element (HREE) contents. Chemical criteria for typical slab-derived magmas are

TABLE 1 Characteristics of magmas

	Characteristics of slab melts	CB ignimbrite	CB granite	Coastal Batholith from mantle wedge
La/Yb	>20	32	71	13.2
Sr/Y	>40	93	67	9
Yb	≤1–1.5	0.68	0.36	1.99
Y	≤15–18	7	6.9	23.9
Al ₂ O ₃	>15%	15.3	15.1	14
Contemporaneous basic rock	very rare	—	very rare	voluminous synplutonic
Na ₂ O		3.97	4.31	3.67

Chemical characteristics of slab melts^{3,16}, Cordillera Blanca (CB) ignimbrites, granites, and mantle-wedge-derived granites of the Coastal Batholith¹⁸.

given in Table 1, where it is evident that the Cordillera Blanca rocks differ from the mantle-wedge-derived³⁰ granitoids of the Coastal Batholith to the west (Fig. 1) and apparently fit the characteristics of slab melts as defined in refs 3 and 16. They lie in the Archaean high-Al TTD and adakite¹⁷ field (Fig. 2), consistent with an origin as partial melts of amphibolite with different proportions of garnet.

Various origins have been suggested for trondhjemites of the TTG suite. Crystal fractionation seems unlikely in many cases, as coexisting basaltic and intermediate rocks are usually lacking^{3,5}. In the Cordillera Blanca batholith, it is impossible to relate the granites to earlier tonalitic rocks, and there are no coeval basic rocks¹². It would in any case be difficult to derive the high Sr/Y, La/Yb ratios and low Y and HREE contents of these magmas by fractional crystallization of the main phases present in the rocks: plagioclase, hornblende and clinopyroxene^{3,12,18}. Nor is it likely that rocks with SiO₂ > 70% could be derived directly from the mantle^{19,20}. Alternative derivations are partial melting of subducted oceanic lithosphere, or

TABLE 2 Major and trace element compositions

	1	2	3	4	5
SiO ₂	70.4	71.6	69.66	69.61	70.4
TiO ₂	0.29	0.27	0.42	0.38	0.29
Al ₂ O ₃	15.27	15.06	15.03	15.10	16.05
Fe ₂ O ₃	0.93	0.58	2.37	2.79	0.79
FeO	0.45	0.97	—	—	1.42
MnO	0.07	0.05	0.03	0.04	0.06
MgO	0.30	0.54	0.83	1.17	0.66
CaO	2.40	1.96	2.02	2.63	2.27
Na ₂ O	3.97	4.31	4.15	4.45	4.84
K ₂ O	3.21	3.51	3.46	2.23	2.59
P ₂ O ₅	0.09	0.08	0.13	0.10	0.09
LOI	2.05	0.62	1.05	0.69	0.79
Total	99.43	99.55	99.15	99.19	100.25
Ba	891	718	—	—	885
Rb	103	120	104	83	76
Sr	652	461	340	351	547
Y	7.0	6.9	9.0	9.0	0.89*

1. Average of seven ignimbrites from Cordillera Blanca.
2. Average of 17 granites from Cordillera Blanca.
3. H50, Kivijarvi TTG grey gneiss ss. Archaean, E. Finland¹⁹.
4. Average of 48 Archaean TTG grey gneisses from E. Finland¹⁹.
5. Average of nine Proterozoic southwest Colorado trondhjemites³⁸.

* Yb.

partial melting of basaltic lower crust; note that partial melting of old Precambrian-type crust is precluded by its absence. For subducted lithosphere to be melted, the primary requirement is that it be hot—in other words, young⁶.

Some Cenozoic magmas are certainly related to young subducting slab and may be the product of slab melting^{6,21}. However, we suggest that slab melting was not involved in producing the Cordillera Blanca magmas. First, there is the age of the slab entering the trench. Numerical simulation of pressure–temperature–time paths of amphibolitic oceanic crust shows that partial melting can occur only during the early stages of subduction initiated in hot, young (<50 Myr) oceanic lithosphere²². The Yb-depleted magmas characteristic of slab melting in post-Cretaceous arcs are found only in young subducted crust (≤25 Myr at the trench⁶). During the period 20–5 Myr ago, the Nazca Plate had passed the initial stage of subduction and was 55–65 Myr old at the trench²³; it was therefore too old and cold for melting to occur before dehydration. Second, models of the thermal structure involving slab-induced convection^{24,25} show slab melting in the corner of the mantle wedge²⁶ ~80 km from the trench. It follows that the TTG arc should be outboard of the main volcanic/plutonic arc, in the arc–trench gap. In Peru the TTG suite lies 300 km inboard of the present trench and volcanic arc¹², and directly above the thickened keel of the Andes¹⁵. Third, lead isotope values lie in a small coherent field (²⁰⁷Pb/²⁰⁴Pb = 15.610–15.635; *n* = 6) similar to the Dupai group and quite different from the trend for mid-ocean-ridge and ocean-island basalts²⁷. The lead values do not lie on mixing curve between the Nazca plate basalt and Pacific sediments, and we follow Mukasa and Tilton in considering that mid-ocean-ridge basalt from asthenospheric mantle²⁷ was not involved. Sr_i (initial ⁸⁷Sr/⁸⁶Sr) and Nd_i (initial ¹⁴³Nd/¹⁴⁴Nd) data (Sr_i 0.705248–0.705571, *n* = 10; Nd_i 0.512611–0.512512, *n* = 10; ref. 28 and N.P., M.P.A. and A. Halliday, manuscript in preparation) are consistent with, but do not prove, derivation from an enriched continental lithosphere similar to that identified in Chile²⁷.

We conclude that rocks of the Cordillera Blanca were not derived directly from the mantle or slab. Instead they may have formed in a manner similar to many Cordilleran batholiths^{29,30}, from crust that is basaltic (density 3.0 Mg m⁻³) and does not include Precambrian basement-type material³¹. We now

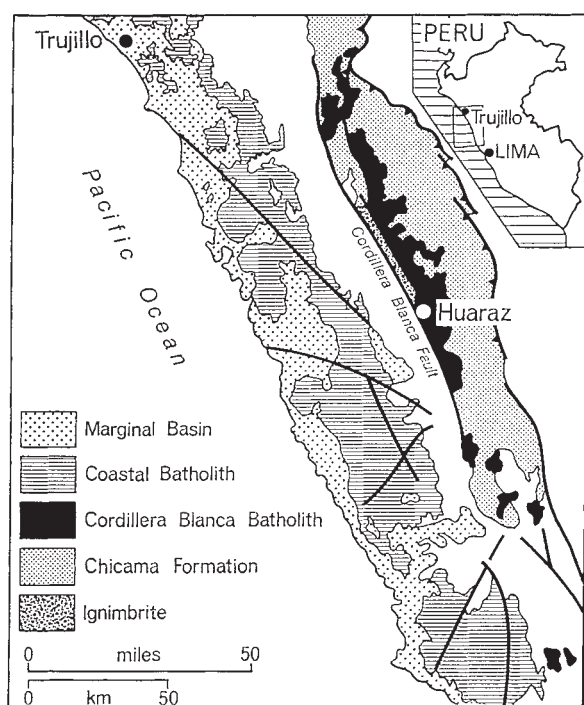


FIG. 1 Simplified map of area north of Lima, Peru, showing the Cordillera Blanca Batholith and ignimbrites lying inboard of the Upper Cretaceous Coastal Batholith, over the deep crustal keel of the Andes.

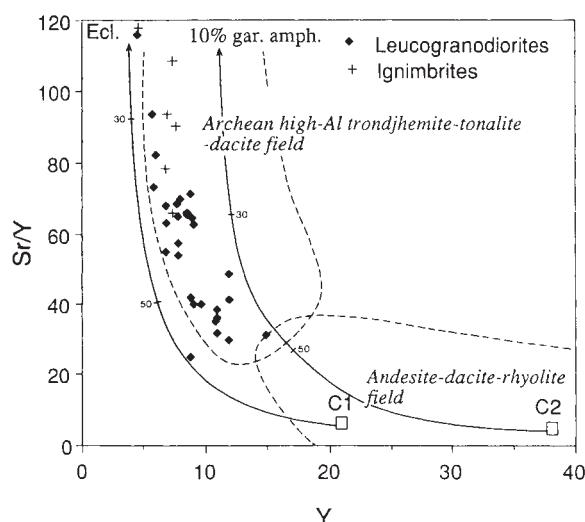


FIG. 2 Plot of Sr/Y against Y for Cordillera Blanca granitic and ignimbritic rocks with Archean and ADR fields³. Partial melting curves for basalt leaving residues of 10% garnet amphibolite (gar. amph) and eclogite (Ecl) from Drummond and Defant¹³. Partial melt values shown on curves. Adakites also plot in TTD field.

consider the thermal state and crustal thickness, to determine the likelihood that the trondhjemites were produced by melting of the lower crust. The thickening of the crust beneath the Cordillera Blanca was by magmatic accretion since the Miocene³¹. This is consistent with high heat flow across Western Cordillera and altiplano³². No tectonic shortening occurred, and the whole of the Western Cordillera is now in isostatic equilibrium, supported by its crustal root and possibly hot underlying mantle³¹. Both the underplating and uplift would heat the crust³³.

Electrical conductivity surveys³⁴ indicate a telluric current channelled into a high-conductivity zone which follows the general trend of the mountain range just to the south of the Cordillera Blanca. The zone lies ~50 km below the present surface and may be associated with a low-velocity zone³⁵ below 40 km. Conductivities are similar to those in the Basin and Range province of North America (0.1 Sm⁻¹), which Bott³⁶ considered could be due to a melt fraction in highly conducting rock. A related feature in James's¹⁵ model for the central Andes is the low shear velocity in the upper mantle (4.25–4.30 km s⁻¹) along the path of the high-conductivity zone. This suggests a hot upper mantle in the same region. High-level thermal features include thermal gradients of 40–60 °C km⁻¹ (ref. 32) and active thermal springs along the line of the Cordillera Blanca fault system.

The data indicate a high geothermal gradient extending from shallow crustal depths down to the mantle, with melt probably present at ~50 km depth. This structure relates to the underaccretion and uplift and has existed since the Miocene, when temperatures in the crust must have exceeded those of the shield geotherm. The lowest temperature gradient estimated from analysis of obducted lower crust³³ is 20 °C km⁻¹; even for this conservative estimate, temperatures at the bottom of the crust (~50 km) would have been greater than 1,000 °C—sufficient to produce trondhjemitic melts from hydrated basaltic compositions with residues of amphibole, garnet and clinopyroxene and little or no plagioclase³⁷. This is consistent with the views of those who consider high-Al TTD magmas to result from partial melting of amphibolite, with or without garnet^{38,39}. Such magmas have high Na₂O and Sr and low HREE contents, and on a Sr/Y versus Y plot (Fig. 3) they lie in the Archean high-Al TTD adakite field (Fig. 2).

Miocene/Pliocene trondhjemite, tonalite and ignimbrite comprise the final magmatic event (~5 Myr) in the central Andes of Peru¹²; we consider that they were produced by partial melting of lower crust that had recently been, or was still being, thickened by magma underplating. We envisage a dynamic model in which compositions of partial melts evolved to reach TTG types as the crust thickened by underplating, and passed through the garnet-in transition about the end of the Miocene.

Other probable examples of melting of thick lower crust include the late Tertiary granitoids of central Chile⁴⁰, trondhjemitic rocks of west central Idaho and north Cascades⁴¹ and the eastern tonalites of the Peninsular Range²⁹. It is not appropriate to use a single model involving slab melting to explain all TTG from the Archean onwards. □

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Inorganic carbon uptake in hydrothermal vent tubeworms facilitated by high environmental $p\text{CO}_2$

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THE marine invertebrate *Riftia pachyptila* has a remarkable symbiosis with intracellular carbon-fixing sulphide-oxidizing bacteria which was first discovered at 2,450 m depth on the Galapagos Rift¹⁻⁴. Such symbiotic arrangements have since been found in a variety of invertebrate taxa and habitats^{5,6}. Studies of these symbioses have focused on temperature, sulphide and oxygen as critical environmental parameters^{5,7-9}. As *Riftia* has a high growth rate and its symbionts are far removed from the host surface^{10,11}, inorganic carbon supply to the symbionts has been recognized as a problem and host mechanisms to concentrate inorganic carbon have been posited^{12,13}. Increased environmental CO_2 partial pressure ($p\text{CO}_2$) has not seriously been considered as a critical environmental parameter^{7,14}. Here we report that elevated $p\text{CO}_2$ (2.9 kPa) in the worms' environment is a determinant of internal total CO_2 (ΣCO_2) and $p\text{CO}_2$, facilitating CO_2 transport and diffusion to the symbionts. We propose that elevated $p\text{CO}_2$ is a potentially critical environmental factor for this species as well as for other chemoautotrophic symbioses.

Although seawater contains an abundance of inorganic carbon in the form of bicarbonate, marine autotrophs with high

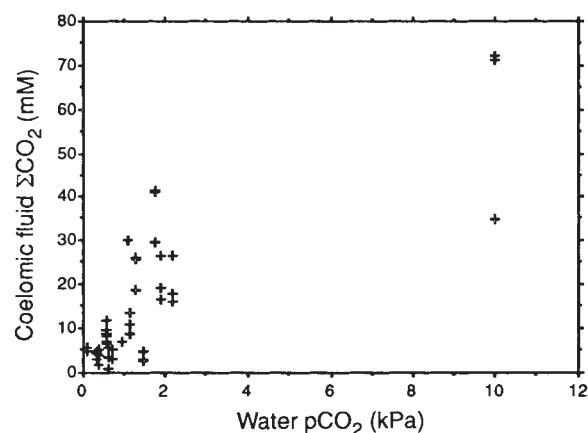


FIG. 1 Relationship between external $p\text{CO}_2$ and coelomic fluid total inorganic carbon, ΣCO_2 , in the hydrothermal vent tubeworm *Riftia pachyptila*. Worms were maintained at 21.4 MPa pressure in flowing water pressurized aquaria for 20 h after capture. Water was buffered with MOPS (3 g l^{-1}) and maintained at a single pH (between 6.17 and 7.76) during an incubation. Water ΣCO_2 was adjusted to values ranging from ambient sea water to 11 mM. Coelomic fluid and blood have nearly identical values of ΣCO_2 , pH and $p\text{CO}_2$ in living worms¹³. Analytical methods are described in Table 1 legend.

rates of carbon fixation have potential problems with inorganic carbon uptake because CO_2 , in the freely diffusible form which is also the species fixed, is normally at low partial pressures owing to its high solubility and the relatively alkaline pH of sea water¹⁵. This situation is exacerbated in chemoautotrophic symbioses because the diffusion path to the symbionts may be relatively long. In *Riftia* this problem is extreme because the symbionts are located far from the animal's surface in a highly vascularized organ, the trophosome^{16,17}. Oxygen, carbon dioxide and sulphide are taken up from the medium into the blood as it passes through the large prominent plume and are transported in the blood to the trophosome^{7,17,18}. Oxygen and sulphide are transported bound to haemoglobins in the blood¹⁸⁻²⁰. Inorganic carbon, however, is not bound by these haemoglobins, as demonstrated by their failure to concentrate it from a carbonate solution during equilibrium dialysis (N.K.S. and J.J.C., unpublished result). The trophosome is located within the trunk of the worm, bathed in coelomic fluid which is in equilibrium with the blood for small molecules such as CO_2 (refs 13, 21).

TABLE 1 Coelomic fluid parameters of *Riftia pachyptila* as a function of treatment

Treatment	n	ΣCO_2 (mM)	$p\text{CO}_2$ (kPa)	pH
1. Removed from vent and analysed the same day	13	31.3 ± 3.4	4.11 ± 1.01	7.18 ± 0.04
2. Removed from vent, recovered at -1.8°C and analysed the same day	3	29.7 ± 5.9		
3. Removed from vent, kept 24 h at 12.2 MPa and low $p\text{CO}_2$ ($<0.05 \text{ kPa}$)				
(A) kept an additional 8 h at 12.2 MPa	2	2.7, 1.3	0.79, 0.71	6.59, 6.63
(B) kept an additional 8 h sealed in tube at 12.2 MPa	2	4.2, 4.5	1.65, 2.48	6.50, 6.45
4. Removed from vent, left on bottom >2 days, recovered and analysed immediately	4	7.0 ± 1.1	1.75 ± 0.13	6.83 ± 0.15
5. Removed from vent, kept 20 h at 21.4 MPa and elevated $p\text{CO}_2$				
(A) ΣCO_2 , 2.0–2.5; $p\text{CO}_2$, 0.11–0.59; pH 7.6–7.8	16	6.4 ± 0.7	1.07 ± 0.15	7.14 ± 0.07
(B) ΣCO_2 , 4.3–6.0; $p\text{CO}_2$, 1.73–2.16; pH 6.5–7.0	9	26.2 ± 3.3	2.86 ± 0.47	7.30 ± 0.13
(C) ΣCO_2 , 11.0; $p\text{CO}_2$, 9.96; pH 6.2	3	59.4 ± 12.2	7.05 ± 0.49	7.44 ± 0.11

Tubeworms were collected from the Genesis vent site at 2,638 m depth at $12^\circ 48.675' \text{N}$, $103^\circ 56.386' \text{W}$ on the East Pacific Rise. One atmosphere is 101.3 kPa or 760 torr; 21.4 MPa corresponds to 2,150 m depth. Except for treatment 2, all were recovered at depth temperature (2.4°C) in an insulating plastic box²¹. Animals in treatment 2 were recovered individually, one each from the Genesis, Parigo and Elsa vent sites, in a slurry of ice and sea water in an insulated box chilled by Peltier effect thermoelectric devices. Animals kept alive at the surface were maintained in pressurized, flowing water aquaria at 8°C and the indicated hydrostatic pressure^{13,29}. Worms in treatment 3B were sealed in their tubes with heavy plastic film held over the open end with rubber bands so that there was no exchange with water in the pressure system. Worms in treatment 5 were maintained in the pressure systems in sea water whose pH and $p\text{CO}_2$ has been modified by bubbling with CO_2 , titration with HCl, and addition of MOPS buffer to a concentration of 3 g l^{-1} . Worms in treatments 3 and 5 were continuously exposed to $\Sigma\text{H}_2\text{S}$ between 100 and 250 μM . ΣCO_2 was measured on 0.25–0.50 ml aliquots with a gas chromatograph^{13,21}. $p\text{CO}_2$ and pH were measured with a Radiometer BMS-3 blood gas analyser at 10°C . Means are shown $\pm$ the standard error of the mean.

TABLE 2 Properties of sea water at the Genesis vent site

Water source	n	T (°C)	pH	ΣCO_2 (mM)	$p\text{CO}_2$ (kPa)	$\delta^{13}\text{C}$ (‰)	$\Sigma\text{H}_2\text{S}$ (mM)	Cl^- (mM)	Mg (mM)
Smoker	2	286	4.06, 4.73	30.81, 25.71	34.52, 19.32	-2.7	4.74, 3.90	501	10.1
Among <i>Riftia</i> tubes	7	19.1 ± 0.14	6.22 ± 0.14	4.70 ± 0.54	2.89 ± 0.64	-1.0 ± 0.8	0.086 ± 0.024	535 ± 7	38.6 ± 0.8
Deep sea water		2.4	7.8	2.3	0.024		0	556, 570	40.7, 41.5
Extrapolated end-member		375	3.51	40.1			5.91	491	

Water samples were collected using titanium samplers either from the *Alvin* or from the *Nautille*. With the *Alvin* (smoker samples and 5-tube samples), the temperature was taken before water sampling and the sample inlet was positioned as close as possible to where the temperature was measured. With *Nautille* (2 'tubes' sample) the temperature at the inlet was measured during sampling. ΣCO_2 and $\Sigma\text{H}_2\text{S}$ were measured on 0.25-ml aliquots with a gas chromatograph^{4,5}. $p\text{CO}_2$ and pH were measured with a Radiometer BMS-3 blood gas analyser at 10 °C. Means are shown $\pm$ the standard error of the mean. Chloride was measured by coulometric titration and magnesium was measured by atomic absorption spectrometry. Ambient water properties other than Cl^- and Mg are from the literature²⁰. Deep sea water $p\text{CO}_2$ was estimated using solubility tables and assuming a temperature of 2 °C and a salinity of 34‰ (ref. 30). 'End-member' composition was estimated by regressing the various parameters against Mg concentration and extrapolating to zero magnesium concentration²⁰.

Observations on the blood and coelomic fluids of freshly recovered *Riftia pachyptila* (2 to 3 h after capture at 2,600 m) have previously shown that blood and coelomic ΣCO_2 concentrations approach 12 mM at 21° N on the East Pacific Rise (EPR)²¹ and 14 mM at the Galapagos Rift¹³. These elevated ΣCO_2 levels were attributed to anaerobic metabolism under the hypoxic conditions prevailing in the closed recovery containers. This conclusion became untenable when we (HERO expeditions 91 and 92 (in October 1991 and April 1992)) sampled *Riftia* from the Genesis site at 13° N EPR. These worms had blood and coelomic fluid ΣCO_2 consistently >20 mM and up to 46 mM (Table 1). The $p\text{CO}_2$ was also extremely high (Table 1). We considered that these blood concentrations must reflect *in situ* levels.

One test of this hypothesis was to slow metabolism in the worms during recovery, which we did by recovering them at the freezing point of sea water using an *in situ* thermoelectric freezer (treatment 2, Table 1). The worms so collected had the same ΣCO_2 , supporting our hypothesis. The second and third tests isolated effects of recovery and hypoxia from vent conditions. The second test evaluated the build-up of ΣCO_2 in *Riftia* kept anoxic within their tubes. Two worms, previously kept for 24 h at the surface, were sealed in their tubes with plastic film and kept in this condition for 8 h before analysis. They showed increases of about 2 mM ΣCO_2 and 1.33 kPa $p\text{CO}_2$, indicating that elevated ΣCO_2 found in freshly captured worms would be only slightly increased if they remained entirely within their

tubes for the 2 to 3 h from capture to analysis (treatment 3, Table 1). The third test consisted of removing tubeworms from the area of active venting, laying them on bare rock a few metres away, out of contact with vent water, and then recovering and analysing them two days later. The four worms exposed to this treatment (treatment 4, Table 1) had greatly reduced ΣCO_2 and $p\text{CO}_2$. Therefore the elevated blood ΣCO_2 is due to the vent field and not the recovery process. Thus, these tests support our hypothesis that blood and coelomic ΣCO_2 and $p\text{CO}_2$ values measured on freshly recovered worms reflect *in situ* levels in the worms.

To determine whether the source of increased internal CO_2 concentrations could be elevated environmental $p\text{CO}_2$, we incubated worms for 20 h at various $p\text{CO}_2$ values. The internal ΣCO_2 and $p\text{CO}_2$ values of these worms reflected the external conditions, indicating that raised external $p\text{CO}_2$ could explain the elevated *in situ* blood values (Fig. 1, Table 1). We had previously dismissed the possibility that high environmental CO_2 was an important factor because measurements of EPR high-temperature vents (smokers) had shown only modest enrichment (3 to 13 mM)²², which after dilution to the warm waters around the worms were presumed not to be significant. Stimulated by the new findings, however, we took water samples at Genesis among the *Riftia* tubes and from a smoker. Water among the tubes was quite high in $p\text{CO}_2$ (2.89 kPa mean, 4.44 maximum) as a result of elevated ΣCO_2 and low pH (Table 2). Worms gain access to sulphide at the concentrations in the water around the tubes because incomplete mixing of venting water brings undiluted water from among their tubes up to their plumes^{9,23} and apparently this applies to CO_2 as well.

Our smoker water samples extrapolated to zero magnesium concentration²² indicate that end-member ΣCO_2 is about 40 mM (Fig. 2), with pH 3.5 at 375 °C and $[\text{Cl}^-]$ of 491 mM. Thus, our data indicate that there has been an oversight on the part of vent biologists with regard to elevated CO_2 at warm vents and on the part of chemists with regard to the ΣCO_2 content of smoker water in this region. Water among the worm tubes represents a 95% dilution of smoker water based on temperature, and a 93.5% dilution based on ΣCO_2 , so no special mechanism is needed to explain the concentrations around the worms. The lower blood ΣCO_2 (10 to 14 mM) documented on the Galapagos Rift and 21° N EPR (refs 13, 21) suggest that *Riftia* at these sites experience lower $p\text{CO}_2$ values because of lower end-member ΣCO_2 values.

We conclude that the very high ΣCO_2 values in freshly collected worms result from exposure to elevated environmental $p\text{CO}_2$. Thus, there is the potential for a substantial gradient driving CO_2 diffusion across the plume and within the trophosome. Although other transport mechanisms may be involved, inorganic carbon movement from the environment into the blood appears to be dominated by CO_2 diffusion. The high concentrations of carbonic anhydrase in plume and trophosome tissue²⁴ can enhance diffusive fluxes, supporting this mechanism. Given

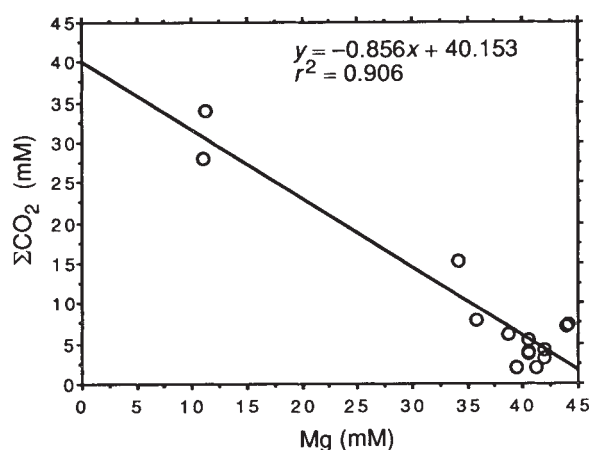


FIG. 2 Relationship between magnesium content and ΣCO_2 in warm and hot vent waters at the Genesis site at 13° N on the East Pacific Rise. ΣCO_2 was analysed by gas chromatography^{13,21} and Mg was analysed by atomic absorption spectrometry. Extrapolation to zero Mg concentration provides an estimate of the ΣCO_2 in undiluted hydrothermal fluids, where magnesium is believed to be almost zero²².

the alkaline pH of blood compared to vent water, ΣCO_2 can be concentrated into the blood (Table 1). Raised ΣCO_2 in *Riftia* blood greatly reduces necessary CO_2 exchange per circuit of the blood from an improbable 33% (ref. 13) to a more supportable 6% at 10 mM, or 2% at 30 mM, further emphasizing the importance of increased environmental $p\text{CO}_2$ for this species.

The unusual stable carbon isotope composition of *Riftia* ($\delta^{13}\text{C} = -9$ to -14% ; refs 8, 25) led to suggestions that the symbionts are carbon-limited^{14,25}, that the fractionation results from a 4-carbon mechanism¹², and that an unusual inorganic carbon source is present in the vent environment²⁶. We found a $\delta^{13}\text{C}$ value of -1.0% for ΣCO_2 around the worm tubes, ruling out unusual source water (Table 2). We also found that total inorganic carbon $\delta^{13}\text{C}$ averaged $-9.6 \pm 3.1\%$ ($\Sigma\text{CO}_2 = 21.6 \pm 1.5$ mM) in coelomic fluids of three Genesis *Riftia*. Assuming CO_2 in these fluids is recently environmentally derived, that is,

not primarily of respiratory origin, these data suggest that some of the fractionation results from diffusion of CO_2 , which is more depleted in ^{13}C than is HCO_3^- , into the worm²⁷. At pH 6.22, the observed pH of the vent fluid, the $\delta^{13}\text{C}$ of CO_2 would be about -5.9% . For fixation to occur with little further fractionation, a limitation of carbon supply to the symbionts, as might be expected at rapid growth rates, or another factor preventing fractionation would be required.

In addition to the importance of elevated $p\text{CO}_2$ for *Riftia*, it is probably necessary for the functioning of other active chemoautotrophic symbioses, most of which occupy environments potentially elevated in $p\text{CO}_2$. One possible example is that elevated ΣCO_2 upon capture has also been found in the blood of the vent clam *Calyplogena magnifica*, raising the possibility that this species also depends on increased $p\text{CO}_2$ (ref. 28). □

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Dark habitats and bright birds illustrate the role of the environment in species divergence

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ANIMALS exhibit a diversity of colour patterns that are commonly used in interspecific and intraspecific communication^{1–3}. Factors influencing the evolution of signals used in communication include the properties of the physical environment in which the signal is generated^{2–10}, the perceptual systems of individuals (such as potential mates or predators) receiving the signal^{2,3,11–16}, and the nature of the information signalled. In warblers of the genus *Phylloscopus*, species differences in colour patterns are correlated with light intensity of the habitat: brighter species live in darker habitats. I report here two observations that colour patterns function to increase conspicuousness, and are used in intraspecific communication. First, individuals make themselves temporarily more conspicuous by flashing the bright colour patterns in display, and are less conspicuous when not displaying. Second, experimentally increasing conspicuousness of males within a given habitat increases territory size, whereas experimentally reducing conspicuousness results in either a smaller territory or its total loss. Traits used in intraspecific communication are often thought to diverge as a result of variation in perceptual systems^{2,3,13,15–17}. This study shows that variation in the physical environment can cause species divergence, and this will occur whether perceptual systems are variable or relatively constant.

Eight *Phylloscopus* species together with the goldcrest, *Regulus regulus*, breed in the forests of Kashmir, India¹⁸. All species are small and greenish, but they differ in plumage patterns: different species have a different number of colour patches on their upperparts (wings, crown, rump and tail)¹⁹. The following patterns are found: no colour patches; a single wing-bar; two wing-bars; two wing-bars plus a crown-stripe; two wing-bars and crown-stripe plus a rump patch; and finally, all of these patches plus white outer tail feathers (Table 1). The distribution of patches across species is nested, in that species with more complex patterns have all the patches of species with simpler patterns. Apart from the white tail, the patches of all species are a similar yellow colour (see for example species with wing-bars in Fig. 1a). Species can therefore be unambiguously ranked from duller to brighter.

Because the ability to perceive visual signals is related to the amount of light in the environment, properties of the micro-habitat should influence the evolution of colours and behaviours used in visual signalling^{2,3,9,10,20}. In *Phylloscopus*, species that breed in dark, dense habitats have a greater number of colour patches than those that breed in open habitats, where they can be visible even in the absence of conspicuous coloration (Fig. 1b). The product moment correlation between habitat brightness and number of colour patches is -0.87 . Significance testing requires formally controlling for phylogeny. A molecular-based phylogeny for these species is available²¹. Sister species often occupy different habitats²¹ and have different plumage patterns, suggesting that historical association is not artificially inflating the association. This is confirmed by application of Felsenstein's statistical method of independent contrasts^{21,22}. The correlation (forced through the origin) between the independent contrasts for habitat brightness and colour is $r = -0.89$, 5 d.f., $P < 0.05$. As an alternative measure of brightness I examined a single

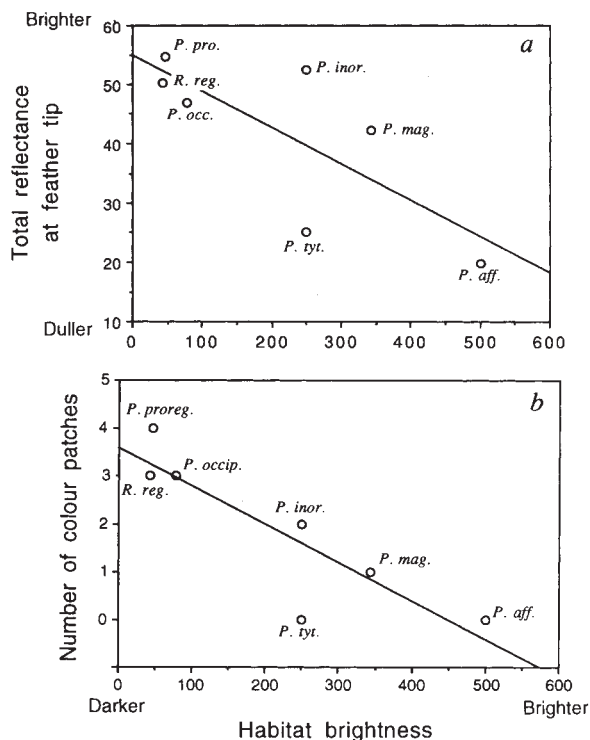


FIG. 1 Association of background radiance (number of foot candles) with a, total reflectance (% relative to a standard) at the tip of the fourth great covert on the wing and b, number of colour patches, exhibited by *Phylloscopus* species and *Regulus regulus* (Table 1). Brightness at the tips of single feathers (a) was measured in the laboratory using a reflectance spectroradiometer. All species except *P. affinis* and *P. tyleri* have unpigmented patches (wing-bars) at this position. Feathers measured were taken from four to ten individuals per species, and the average plotted. I quantified habitat brightness (in Kashmir in May 1991) using a still camera, by holding aperture and film speed constant, and taking automatic shutter speed readouts (a photometric measurement). Shutter speeds were then converted to foot candles as a measure of illumination²⁷. Photometric measurements underestimate radiance at low and high wavelengths relative to middle wavelengths⁶. But most backgrounds in the Kashmir environment are shades of grey, brown, or are rich in middle wavelengths (yellow-green), so a photometric approximation to radiance should be accurate enough for comparative purposes^{6,10}. Habitat brightness was recorded every 50 m from above treeline at 3,600 m to the middle of the coniferous forest at 2,400 m. I took four brightness readings (facing North, South, East, West) at each recording stop, taking care to point the camera across the largest possible open space in order to generate readings as consistent as possible across the different habitats (pointing the camera at a nearby tree, for example, would have produced an abnormally dark reading; pointing the camera toward an individual bird above would have produced a reading dependent on the amount of bright sky visible through the vegetation). I recorded which species were singing within 50 m. I obtained a mean level of habitat brightness for each of the different species as the average brightness across locations in which the species were recorded.

patch position (that of the greater covert on the wing), and measured total reflectance using reflectance spectroradiometry⁶. Species without wing-bars live in the more open habitats (Fig. 1a). The correlation between brightness of the feather tip and habitat was $r = -0.76$ (correcting for phylogeny $0.05 < P < 0.1$).

The habitat-conspicuousness correlation may arise in several ways. For example, the bright species have the most stripes and, in certain habitats, these may be more difficult to see: the association thus may entirely reflect predation pressures²³. Second, there may be a trade-off across species between song and conspicuousness, such that species whose songs are transmitted through the environment less effectively evolve brighter coloration²⁴. Finally, if visibility of colour patterns depends on the background in which they are seen²⁻¹⁰, and colour patterns are used in intraspecific communication, species in darker habitats may evolve brighter coloration to enhance visual display. This last hypothesis has the most direct support.

Variation in display is associated with the location of individual colour patches on the body. For example, species with wing-bars move their wings more (and more often) than species that do not have wing-bars; species that have crown-stripes tip their heads in display, whereas those without crown-stripes do not, and *P. proregulus*, which has a rump patch, turns and flashes its rump (Table 1, Fig. 2). Display movements enhance visibility of the patches: individuals temporarily increase conspicuousness by flashing bright colour patches in display, but are more cryptic when not displaying.

Movement is used temporarily to enhance visibility during display, and species differences in conspicuousness are associated with the background in which signals are transmitted. If variation in conspicuousness is shaped by properties of the environment, then permanently altering conspicuousness (size or number of colour patches) of individuals within a given habitat, while allowing display to remain constant, should affect

FIG. 2 *Phylloscopus* species and *Regulus regulus* exhibiting some of the different movements used in territorial display. Drawings are reproduced from videotapes taken in the field. *P. affinis* (a) has no colour patches, and exhibits no stereotypical display movements. *P. pulcher* (b) has wing-bars and is shown flashing its wings in display. It also has white outer tail feathers which are not used in display, but are conspicuous during flight (d). *Regulus regulus* (c) is shown tipping its head side-to-side in display, approaching the source of the intruder with the head down and the crown-stripe highly conspicuous. *P. proregulus* (e) has a bright rump patch, and turns away from the source of the intruder, flashing the rump.

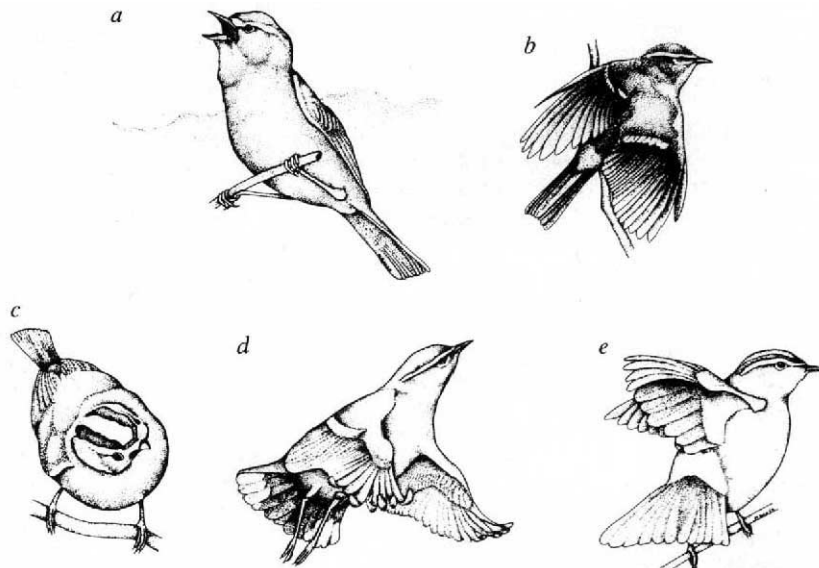


TABLE 1 Association of display movements and colour patch location

Species	Colour patch location	Head tip (degrees)	Rump dipping	Body position	Wings	Body turns (degrees)	N
<i>P. affinis</i>	none	0	0	0	0	0	6 (8)
<i>P. trochilus</i>	none	0	0	0	0.53 (0.13)	3 (1)	4 (6)
<i>P. tyleri</i>	none	0	0	0	0.34 (0.19)	44 (27)	3 (7)
<i>P. collybita</i>	none	0	0	flat	0.33 (0.13)	8 (2)	4 (5)
<i>P. trochiloides</i>	gc	0	0.19 (0.06)	flat	0.79 (0.20)	32 (16)	4 (7)
<i>P. magnirostris</i>	gc	0	0.27 (0.02)	0	1.03 (0.08)	111 (12)	5 (5)
<i>P. inornatus</i>	gc, mc	0	0	0	0.68 (0.15)	42 (16)	3 (15)
<i>P. occipitalis</i>	gc, mc, cr	53 (11)	0.56 (0.26)	0.11 (0.05)	0.62 (0.08)	54 (8)	5 (8)
<i>R. regulus</i>	gc, mc, cr	63 (7)	0.27 (0.02)	0.54 (0.09)	0.72 (0.17)	90 (12)	3 (5)
<i>P. proregulus</i>	gc, mc, cr, r	49 (19)	0.32 (0.06)	0.44 (0.06)	0.84 (0.02)	119 (33)	3 (4)
<i>P. pulcher</i>	gc, mc, cr, r, t	30 (12)	0	flat	0.80 (0.04)	42 (16)	3 (4)

Association of display movements (reflecting interspecific differences in the amount of movement of general areas of the body) and colour patch location in *Phylloscopus* species and a closely related species of a different genus, *Regulus regulus*. Male territorial displays were recorded by attracting them close to the videocamera through the use of song playbacks, and quantified from videotapes played in slow motion on a video analyser. I selected individuals that were actively displaying, and took average values from 1–12 display sequences per individual. The 'colour patch location' category lists areas of the body in which a species exhibits a colour patch¹⁹: none (no colour patches), gc (greater covert wing-bar), mc (median covert wing-bar), cr (crown-stripe), r (rump patch), t (tail patch). The behaviours listed correspond to the areas of the body used in display (Fig. 2): 'head' refers to the angle at which the head is tipped side-to-side; 'rump dipping' is the distance through which the rump is bobbed down and up; 'body position' refers to the postures used in display, for example, approaching with the head down and lower than the rump (positive values), or with the body low and flat (head even with rump); 'wings' is the distance through which the wings are opened in display; 'body turns' is the angle through which the entire body is moved side-to-side in display. Mean amount of movement for each species is indicated numerically, with standard errors in parentheses; zeros indicate that a behaviour is not exhibited. 'Head tip' and 'body turns' are measured in degrees, and the other behaviours are distance standardized by length from top of head to breast. Sample size, *N*, lists the number of individuals of each species in which display behaviour was measured; total number of individuals videotaped is in parentheses (individuals videotaped all behaved similarly; those not measured were too distant from the camera for accurate quantification). The European species (*P. collybita*, *P. trochilus*) were videotaped in England (Cambridge and Sussex) during April, 1991.

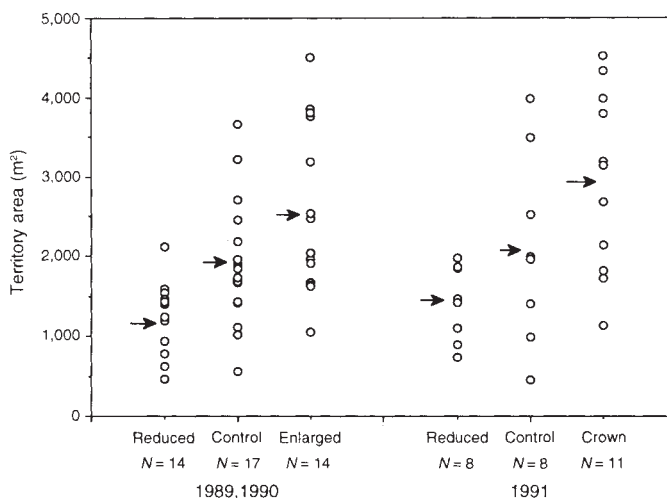
fitness. In 1989 and 1990, I manipulated male coloration in one species, *P. inornatus*, which has two wing-bars but no other colour patches. The manipulation consisted of capturing and randomly assigning males to one of three groups. The first group (control) had its wing-bars painted with clear paint but otherwise unchanged. The second group (reduced) had its wing-bars reduced using green paint, and the third group (enlarged) had its wing-bars enlarged using yellow paint. Manipulations were done while males were establishing territories, and before females arrived on the breeding grounds.

Experimentally altering conspicuousness directly affected territory size in *P. inornatus*. In 1989 and 1990, males with experimentally enlarged wing-bars obtained larger territories than controls which, in turn, obtained larger territories than males with reduced wing-bars (Fig. 3). Size of colour patches affects territory size through intermale interactions. Males were observed to display to each other at close range (≤ 1 m) and for prolonged periods of time (30–60 min). Shifts in boundary location occurred in the days immediately following these inter-

actions and, in each case ($N = 3$), the more conspicuous male gained a portion of a duller male's territory. Song is not used in these displays, suggesting that assessment occurs on the basis of other characters, such as wing-bar size.

It is possible that wing-bar size *per se* affects territory size in *P. inornatus*, because it may indicate dominance²⁵. To test this, in 1991 I added a novel colour pattern (crown-stripe) to *P. inornatus* males, but did not enlarge their wing-bars. This had the same effect on territory size as did the previous manipulations (Fig. 3), indicating that conspicuousness has the main effect on territory size. Experimentally decreasing conspicuousness reduced mating success. Across all years, a larger proportion of males with reduced conspicuousness (27%), relative to control and brightened males (4%), were unable to maintain territories in the area, and did not obtain mates ($\chi^2 = 9.25$, d.f. = 1, $P < 0.01$; total number of males reduced was 30, control 25, and enlarged 27). This suggests that within a given habitat some minimal level of conspicuousness is necessary to maintain a territory and reproduce. But increased conspicuousness did not increase

FIG. 3 Territory areas (square metres) for manipulated and control males. Arrows indicate means for each group. Colour patches were altered using yellow (enlarged), green (reduced) or clear (control) acrylic paints, which remained on the plumage throughout the breeding season (control males were not painted in 1991). Reflectance spectra of the yellow paints were examined (average total reflectance across all years was 17.3%) and found to be lower than that of wing patches (Fig. 1a). It therefore appears that the area of the yellow patch affects territory size, regardless of variation in total reflectance. To measure territories, 1,000 trees over a 20 hectare area were numbered and mapped²⁸. Territory size was recorded by following individual colour-ringed males. Only those trees in which a male was observed singing were considered to be within the territory²⁸. Whether existing colour patches were enlarged (both 1989 and 1990), or whether a novel colour patch was added without enlarging existing colour patches (1991), males whose conspicuousness was experimentally increased obtained larger territories than controls, which obtained larger territories than males whose conspicuousness was experimentally decreased (in 1989 and 1990 combined: Kruskal-Wallis test, $H = 16.5$, $P < 0.01$, mean territory size of males with reduced wing-bars = $1,214 \text{ m}^2 \pm 120 \text{ s.e.}$, mean control = $1,900 \pm 189$, mean enlarged = $2,569 \pm 284$; in 1991: $H = 7.8$, $P < 0.05$, mean territory size of males with reduced wing-bars = $1,413 \pm 166 \text{ s.e.}$, mean control = $2,095 \pm 425$, mean with added crown = $2,946 \pm 345$).



mating success in *P. inornatus*. Males from all experimental groups that maintained territories paired up monogamously and had similar reproductive success (*F*-tests comparing the three groups of males for clutch size and date of first egg in each of the three years, all $P > 0.1$).

Hypotheses explaining species divergence in signalling characters have typically relied on mechanisms such as variation in perceptual systems (such as cues used in female preference or male competition)^{2,3,12,13,16}, or constraints on males' ability to exploit biases in female sensory systems^{2,3,12-15,17}. Under these mechanisms, the sensory system predisposes the evolution of particular male characters, resulting in the evolution of the male trait³. Because conspicuousness is context-dependent, properties

of the environment will bias the direction in which signalling characters evolve (these processes are termed 'sensory drive' by Endler)^{3,13}. The joint evolution of signals, such as colour patterns, with habitat choice and visually directed behaviours (such as courtship display and predation) can create and maintain differences among populations in suites of these traits, ultimately leading to species divergence^{3,13}. Properties of the environment can therefore lead to species divergence whether or not perceptual systems are variable. It is widely accepted that habitat characteristics influence the evolution of cryptic coloration²⁶. Likewise, as this study demonstrates, properties of the environment may strongly influence the evolution of conspicuous coloration. □

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The *torso* receptor localizes as well as transduces the spatial signal specifying terminal body pattern in *Drosophila*

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SPECIFICATION of the end portions of the *Drosophila* body depends on the *torso* (*tor*) protein, a receptor tyrosine kinase that accumulates uniformly along the entire surface of the embryo but is activated only in the vicinity of the poles¹⁻⁶. Several genes are normally required for activating *tor* and appear to define a system in which a gene product tethered to the extracellular vitelline membrane at each end of the egg provides a local source for an extracellular *tor* ligand^{2,5-7}. This ligand would have to diffuse from the membrane to the cell surface of the embryo without losing its spatial localization. Here we report that the failure to accumulate *tor* protein at one or both poles leads to spatially inappropriate activity of more centrally located receptor. This ectopic activity depends on the same gene functions normally required for activating *tor*; thus we infer that it reflects inappropriate diffusion of the ligand to more central regions of the body. We conclude that the receptor not only transduces the spatial signal imparted by the *tor* ligand, but also ensures its correct localization by sequestering the ligand. Ligand trapping by receptor may also localize spatial signals in other patterning systems, including specification of the dorsal-ventral axis in *Drosophila* and of vulval cell fates in *Caenorhabditis elegans*.

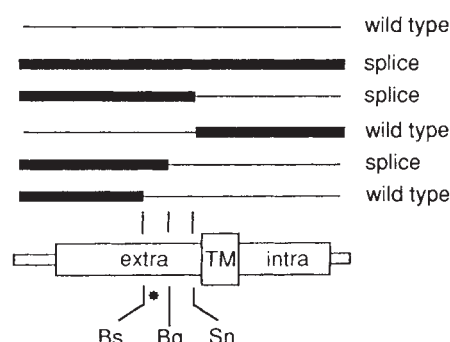


FIG. 1 The *tor*^{RL3} mutation maps to the extracellular domain. Schematic representation of the hybrid *tor-tor*^{RL3} constructs used to map the region responsible for the splice phenotype. A genomic *EcoRI* fragment of around 12 kilobases (kb) derived from wild-type DNA (thin bar) completely rescues the *tor* mutant phenotype when returned to the genome by P-element-mediated transformation⁶. Similarly, the homologous *EcoRI* fragment derived from *tor*^{RL3} DNA (thick bar) behaves like a fully functional copy of the mutant gene (the transformed mutant DNA can rescue the *tor* mutant phenotype, but shows evidence of temperature and dose-dependent ectopic activity as described previously for the endogenous mutant allele^{2,3,6}). Reciprocal 5' and 3' portions of the *tor*⁺ and *tor*^{RL3} DNAs were then combined as shown, transformants obtained, and the rescuing and splice activities associated with at least three independent lines of each hybrid gene assayed by standard means⁶. The lesion maps unequivocally to a *BstXI*-*BglII* fragment (positions, 1,642 and 2,407; numbers according to ref. 5). Sequencing of this fragment revealed a single change in position 1,956 which results in a substitution of leucine for histidine at amino acid 242 in the extracellular domain of the protein. Restriction enzyme analysis of the *tor*^{RL3} DNA revealed a second sequence alteration (the loss of an *Xho* site) in a neighbouring fragment of the gene which we subsequently determined to be a C to A change at position 2,460. This change results in a proline to histidine substitution at amino acid 387 at the junction between the extracellular and transmembrane domains. But hybrid genes which carry this mutation, but not the A to T change at position 1,956, appear functionally indistinguishable from wild type. Bg, *BglII*; Bs, *BstXI*; Sn, *SnaBI*.

The *tor* gene was identified by recessive, loss-of-function mutations which cause embryos derived from mutant females to develop into larvae lacking both the anterior and posterior ends of the body pattern¹. However, several unusual mutant alleles were also obtained that cause a reciprocal phenotype, namely the expansion of terminal portions of the body at the expense of the middle body segments: the 'splice' phenotype²⁻⁴. Several lines of evidence indicate that these mutations cause spatially indiscriminate, constitutive activity of the *tor* protein^{2,3,6,7}. The constitutive activity of one of these mutant proteins, *tor*^{RL3}, is caused by a single amino-acid change in the extracellular domain (Fig. 1), supporting the idea^{5,6} that the receptor is normally activated by a change in the extracellular domain caused by interaction with an extracellular ligand.

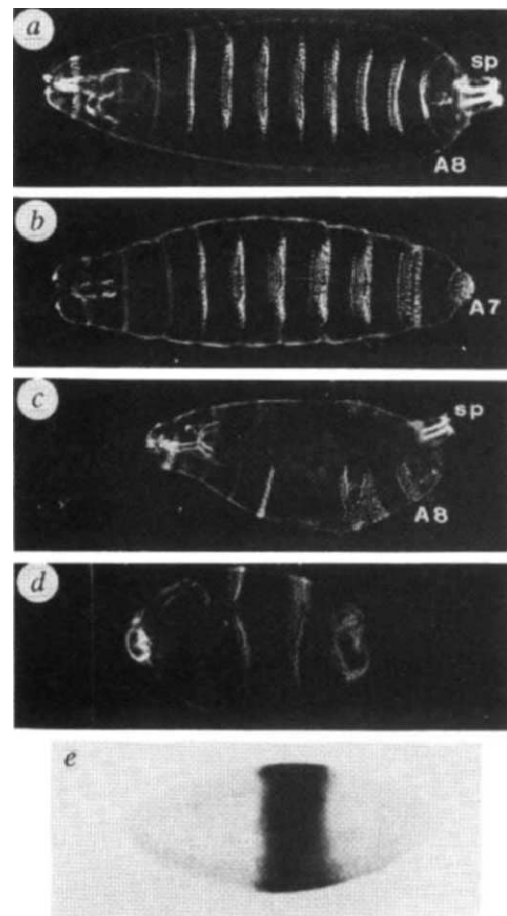
In the first series of experiments, we altered the normal, ubiquitous expression of *tor* protein by generating embryos in which *tor* protein derives from *tor* messenger RNAs containing *cis*-acting response elements that mediate translational repression by the posterior determinant *nanos* (*nos*). The inclusion of these '*nos* response elements' (NREs) causes *tor* protein expression to decline in a graded fashion in the posterior half

of the body⁸. As shown in Table 1 and Fig. 2c, embryos in which *tor* protein derives solely from *tor*^{NRE} transcripts frequently lack middle body segments, a phenotype similar to the 'splice' phenotype caused by the constitutive *tor* mutation *tor*^{RL3}. This phenotype is suppressed in embryos that derive *tor* protein from both *tor*⁺ as well as *tor*^{NRE} transcripts (Table 1), indicating that it results from downregulation of *tor* protein expression by *nos*.

The splice phenotype observed in *tor*^{NRE} embryos depends on the function of *tailless* (*tl*), a downstream gene that mediates the zygotic response to *tor* (Table 1). In this respect it appears identical to the splice phenotypes caused by mutations such as *tor*^{RL3} which cause constitutive *tor* activity^{2,3,9}. However, *tor* protein derived from *tor*^{NRE} transcripts is not constitutively active. Instead, its ectopic activity in *tor*^{NRE} embryos depends on the same upstream gene functions *trunk* (*trk*) and *torso-like* (*tsl*) which are normally required for activating *tor* protein and have been implicated in the local production or action of the *tor* ligand (Table 1; refs 6,7). Hence, inadequate posterior accumulation of *tor* protein in *tor*^{NRE} embryos seems to lead to ligand-dependent activation of the receptor in more central regions. We therefore infer that in wild-type embryos,

FIG. 2 Defects in the middle body segments in *tor*^{NRE} and *Kr-tor* embryos. Ventral aspects of embryos derived from wild type, (a), *tor*^{XR1} (b) and *tor*^{XR1} *P(tor^{NRE})#2/tor^{XR1}* (c) females, and of *P(Kr-tor)* embryos derived from *tor*^{XR1} females (d). e, *tor* transcripts in a *Kr-tor* embryo during nuclear cycle 14. Note the lack of terminal structures such as A8 and the posterior spiracles (sp) in *tor*^{XR1} embryos (b), and conversely, the loss of middle body segments in *tor*^{NRE} (c) and *Kr-tor* (d) embryos. Note also that *tor* transcripts in the *Kr-tor* embryo (e) are expressed in the central portion of the body, like the endogenous *Kr* gene. The phenotype of the *tor*^{NRE} embryos differs from that of the *Kr-tor* embryos in two respects. First, *tor*^{NRE} embryos always form at least some posterior terminal structures as filzkörper and posterior spiracles, whereas *Kr-tor* embryos do not. Hence, even though no *tor* protein expression was detected at the posterior pole in *tor*^{NRE} embryos⁸, we infer that the *nos*-dependent downregulation of *tor* protein expression in *tor*^{NRE} embryos is not complete and that the residual low level of *tor* receptor expression at the posterior pole can mediate the development of terminal structures such as the posterior spiracles. However, the level of posteriorly situated receptor appears in these embryos to be too low to prevent ligand-dependent ectopic activity of more centrally located receptor (Table 1). In contrast, virtually all embryos derived from *tor*^{XR1} females that carry two copies of the *P(tor^{NRE})#2* insertion develop normally (data not shown). Hence, a twofold increase in the maternal *tor*^{NRE} gene dosage allows sufficient receptor to accumulate at the posterior pole to block inappropriate diffusion of the *tor* ligand to more central portions of the embryo. As shown in ref. 8, embryos derived from *tor*^{WK} or *tor*^{PM} females carrying a single copy of the *tor*^{NRE} gene do not form posterior spiracles or other posterior terminalia; nor do they show any evidence of ectopic *tor* activity (see also Table 1). In these embryos, low levels of posteriorly situated *tor*⁺ protein which derives solely from *tor*^{NRE} transcripts must compete with wild-type levels of *tor*^{PM} or *tor*^{WK} protein⁶ to interact with limiting amounts of the *tor* ligand. Under these conditions, the mutant protein appears to preclude productive ligand-receptor interactions involving the wild-type protein. The second difference between *tor*^{NRE} and *Kr-tor* embryos is that the latter show evidence of ventralization as well as loss of middle body segments. Although this aspect of the *Kr-tor* phenotype is unexpected, our finding that both the segmentation and ventralization phenotypes are similarly suppressed by the presence of ubiquitously expressed *tor*⁺, *tor*^{PM} or *tor*^{WK} proteins and by mutations in the *trk* gene (Table 1) provides further evidence that absence of the *tor* receptor at the poles leads to ectopic activity of more centrally located receptor. One possible explanation for the unexpected ventralization phenotype is suggested by recent evidence of overlaps between components of the signalling systems mediating the dorso-ventral and terminal signals in early embryos^{15,16}. Because zygotic *Kr-tor* transcripts arise significantly later than the maternal *tor*^{NRE} transcripts, *tor* protein derived from *Kr-tor* transcripts is likely to appear much later than *tor* protein derived from maternal transcripts. This temporal difference might allow *tor* activity to be transduced inappropriately by downstream components of the dorso-ventral signalling system.

METHODS. The *P(tor^{NRE})* gene has been described previously (*tor(hbl+hb2)*; ref. 8). The *Kr-tor* gene was constructed by placing a 6 kb fragment from



the upstream regulatory region of the *Kr* gene (from the *Xba*I site at about -7 kb to the *Sall* site at -1 kb; ref. 10) upstream of the *hsp70* promoter (from the *Nru*I site at -45 bp to an artificial *Kpn*I site created at +250 bp) and using this *Kr*-like promoter to drive expression of an 8 kb fragment of *tor* genomic DNA encoding the complete protein (from the *Xmn*I site at 869 to the *Eco*RI site downstream of the coding sequence). *tor* transcripts in *Kr-tor* embryos were visualized using digoxigenin *in situ* hybridization¹⁷. Because the *tor*^{XR1} mutation prevents the accumulation of endogenous *tor* mRNA⁵, the transcripts observed in the *Kr-tor* embryo shown in e derived solely from zygotic expression of the *P(Kr-tor)* gene.

ligand-receptor interactions occurring at the posterior pole normally prevent diffusion of the ligand to more central regions.

A distinct role for the receptor in limiting ligand diffusion is also supported by the existence of mutations that separate this role from signal transduction. As shown in Table 1, the *tor^{NRE}*-associated splice phenotype is also suppressed in the presence of *tor* protein derived from the recessive mutant allele *tor^{PM}*. *tor^{PM}* mutant protein accumulates uniformly along the surface of the early embryo⁶ and is thus appropriately positioned to block diffusion of the ligand even though it cannot transduce the terminal signal. Similar results have been obtained with a second mutation *tor^{WK}*.

The role of *tor* in localizing the terminal signal was also examined in a second series of experiments in which localized zygotic transcription, rather than *nos*-dependent translational control, was used to alter the normal distribution of *tor* protein. In this case, we generated embryos in which *tor* protein derives solely from *tor* transcripts that are expressed in central portions of the body under the control of the *Kr* gene promoter¹⁰ (Fig. 2e). Such embryos frequently lack middle body segments (Table 1; Fig. 2d). Moreover, as in *tor^{NRE}* embryos, we find that these defects are suppressed by the *trk* mutation as well as by the presence of maternally derived *tor⁺* or *tor^{PM}* or *tor^{WK}* protein (Table 1, and data not shown). Hence, as in *tor^{NRE}* embryos, centrally located *tor* protein derived from the *Kr-tor* transcripts

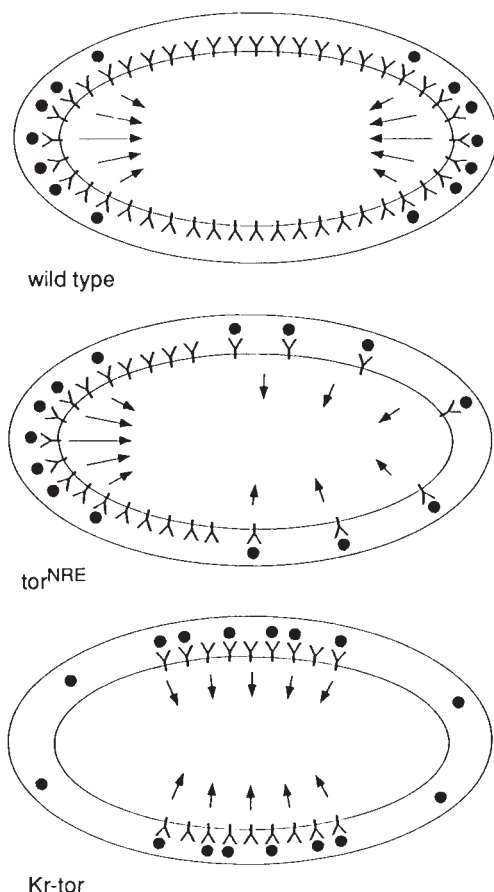


FIG. 3 Schematic diagram of the local activation of the *tor* receptor. The distribution of active *tor* receptor depends both on the local generation of a diffusible ligand at each pole and on the ability of the *tor* protein to trap the ligand, thereby impeding its diffusion. In a wild-type situation this mechanism leads to the activation of the *tor* receptor only at the poles of the embryo. When the *tor* protein is at low levels in one pole (as in *tor^{NRE}* embryos) or is absent from both poles (as in *Kr-tor* embryos), the ligand is able to diffuse further and activates the *tor* receptor in more central parts of the embryo.

TABLE 1 Pattern defects in middle body segments produced by ectopic *torso* activity

Maternal genotype	Developed embryos	Embryos showing middle-body defects
<i>tor^{XR1}; P(tor^{NRE})^{#9/+}</i>	124	75 (61%)
<i>tor^{XR1/+}; P(tor^{NRE})^{#9/+}</i>	112	2 (2%)
<i>tor^{XR1}; P(tor^{NRE})^{#9/tll¹}</i>	49 (<i>tll¹/tll¹</i>)	0
<i>trk tor^{XR1}; P(tor^{NRE})^{#9/+}</i>	133	0
<i>tor^{XR1}/tor^{PM}; P(tor^{NRE})^{#9/+}</i>	191	6 (3%)
<i>tor^{XR1} P(tor^{NRE})^{#2}/tor^{XR1}</i>	196	117 (60%)
<i>tor^{XR1} P(tor^{NRE})^{#2}/tor^{XR1}; tsl</i>	261	16 (6%)
<i>tor^{XR1}; P(Kr-tor)^{#7}</i>	55	39 (71%)
<i>trk tor^{XR1}; P(Kr-tor)^{#7}</i>	104	4 (4%)
<i>tor^{PM}; P(Kr-tor)^{#7}</i>	90	7 (8%)
<i>tor^{XR1/+}; P(Kr-tor)^{#7}</i>	158	3 (2%)

tor^{NRE} embryos were scored as having middle-body patterning defects when the ventral dentical bands associated with one or more abdominal segments were deleted (about 40% of these abnormal embryos show fusions or deletions in three or more abdominal segments). In addition to lacking middle-body segments, *Kr-tor* embryos also showed evidence of ventralization (expansion of the ventral dentical pattern and corresponding loss of the dorsal hair pattern; Fig. 2). These embryos were scored as having middle-body defects when one or more abdominal segments were either missing or ventralized. Most of the *Kr-tor* embryos scored as having middle-body defects showed both segmentation and ventralization phenotypes (about 70% of these embryos have five or fewer abdominal segments). Curiously, the majority of the eggs derived from homozygous *tor^{XR1}*; *Kr-tor* females fail to be fertilized or to develop past the first few nuclear division cycles, in contrast to eggs derived from *tor^{PM}/tor^{XR1}*; *Kr-tor* or *trk-tor^{XR1}*; *Kr-tor* females. Several independent insertions of the *P(tor^{NRE})* and *P(Kr-tor)* genes were generated and used in these experiments; with the exception of a few *P(Kr-tor)* insertions which had no detectable activity, only minor quantitative variations were observed when different insertions of the same gene were tested in the different genetic backgrounds shown. *tll¹/tll¹* progeny derived from *tor^{XR1}; P(tor^{NRE})^{#9/tll¹}* females were recognized by their failure to form both normal anterior and posterior terminalia (for example posterior spiracles which are at least partially formed by *tll⁺* progeny of *tor^{XR1}; P(tor^{NRE})^{#9/+}* females). The *tor^{XR1}* mutation is a small deletion that prevents the accumulation of detectable levels of *tor* transcripts⁵ and its use in these experiments is critical to eliminate *tor* protein expression derived from the endogenous gene.

can be ectopically activated in a ligand-dependent fashion, provided that *tor* protein is not expressed at the poles.

We previously showed that polarized activity of the *tor* receptor leads to the generation of a graded signal which organizes terminal body pattern⁶. Our current results, summarized in Fig. 3, indicate that the distribution of active *tor* protein depends both on the local generation of a diffusible ligand at each pole and on the ability of the *tor* receptor to trap the ligand, thereby impeding its diffusion. Hence, the *tor* receptor not only transduces the terminal signal, but also plays a significant role in ensuring its correct spatial localization.

The *tor* ligand may be localized by being tethered to the terminal regions of the vitelline membrane before fertilization, after which it is released in a freely diffusible form (for example, by proteolytic cleavage). Alternatively, it may be secreted in an inactive form into the perivitelline fluid layer and then activated by a processing system which is locally tethered to the vitelline membrane. Consistent with these ideas, preliminary results suggest that the product of the *trunk* gene could be a secreted inactive form of a signal for the terminal system (our unpublished results). Because of the involvement of the receptor in limiting diffusion of the ligand, both the timing of ligand processing (or released) as well as the amount of ligand generated are likely to be significant factors in controlling the distribution of activated receptor, and hence the generation of the terminal body pattern.

Trapping of a diffusible ligand by its receptor may be a common mechanism for restricting the distribution of a spatial

signal. Dorso-ventral patterning in *Drosophila* depends on an extracellular ventralizing activity which can diffuse freely within the perivitelline fluid of early embryos in the absence of the *Toll* receptor¹¹. Hence, *Toll* may normally trap the ligand and thereby ensure its correct localization. A similar mechanism may also be involved in cell fate specification in *Caenorhabditis elegans*. Mutations that inactivate the putative EGF-receptor homologue encoded by the *let-23* gene block vulval cell induction by the anchor cell¹², whereas mutations that reduce but do not eliminate *let-23* function cause multivulval phenotypes¹³. Multivulval phenotypes also result when the *let-23* ligand, *lin-3*, is overexpressed in the anchor cell¹⁴. We suggest that the hyperinducing *let-23* mutations cause abnormally low levels of the receptor, and that this condition, or abnormally high levels of the ligand caused by *lin-3* overexpression, may allow the ligand to diffuse beyond its normal limit, leading to the multivulval phenotype. Thus, as in the case of the *Drosophila* terminal system, ligand-receptor interactions in these other systems may be involved in localizing as well as transducing the spatial signal. □

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Growth factor function of vasoactive intestinal peptide in whole cultured mouse embryos

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FACTORS controlling central nervous system (CNS) growth immediately after neurulation are mostly unknown. Vasoactive intestinal peptide (VIP) receptors are widely distributed in the embryonic nervous system^{1,2}, and VIP has trophic and mitogenic properties^{3,4} on embryonic neural tissues but inhibits growth⁵ and mitosis in certain tumours⁶. To address the potential effects of VIP on embryonic growth, we used whole postimplantation embryo cultures^{7,8}. After a 4-h incubation, VIP stimulated growth, increasing somite number, embryonic volume, DNA and protein content, and number of cells in S-phase. A VIP antagonist^{9,10} substantially inhibited these VIP-mediated increments in growth. The VIP antagonist completely suppressed VIP-stimulated mitosis in the

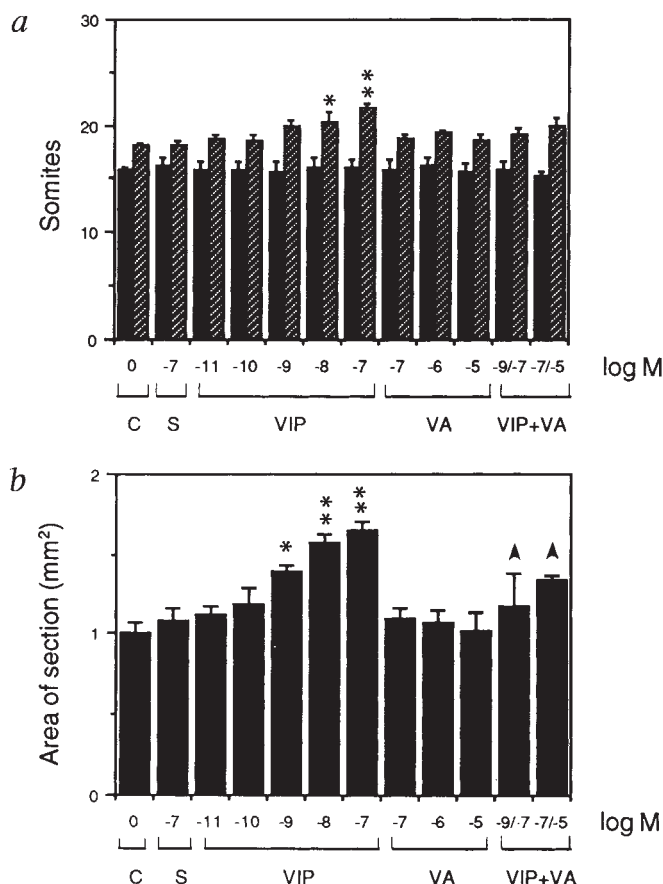
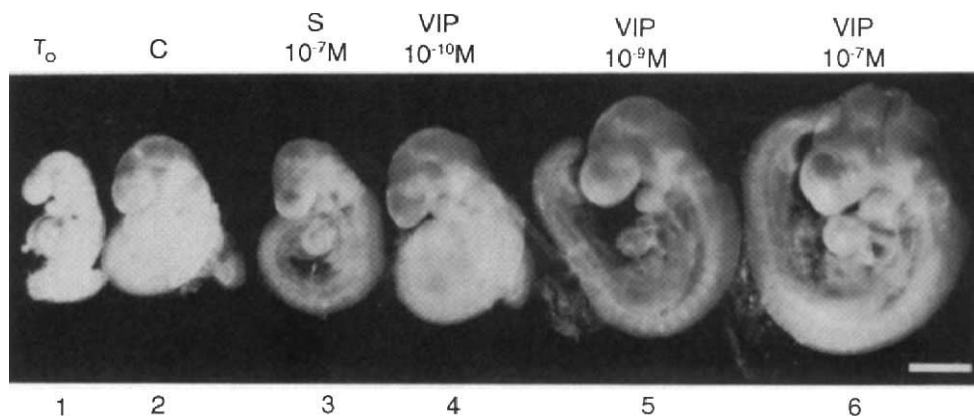


FIG. 1 VIP stimulates the growth of whole cultured embryos when compared with medium alone (C) or secretin (S). In each group, a total of 5-18 embryos that had been processed in at least three separate experiments were included. Bar represents mean $\pm$ s.e.m.; asterisks indicate difference from control (* P < 0.05, ** P < 0.01 or less in ANOVA); $\blacktriangle$, differs from the group treated with a similar concentration of VIP but without VA ($\blacktriangle$, P < 0.05). VA, a hybrid peptide that contains a portion of neurotensin and a portion of VIP, was synthesized and purified as previously described^{9,10}. Previous studies have shown that this antagonist selectively inhibits VIP receptors from CNS versus those in non-CNS tissues²⁷. *a*, VIP increases the number of somites although all groups exhibited identical numbers of somites at the beginning of the culture (see selection criteria below). $\blacksquare$, Before culture; $\blacksquare$, after culture; log M is log of molarity. *b*, VIP dramatically increases the area of section of cultured embryos. The measurement of the area of embryonic section was preferred to the crown-rump length because of the variation of the embryonic curvature. Preliminary work with 14 to 28 somite embryos showed a linear correlation between number of somites and area of section (data not shown). As described by New⁷ and modified for the mouse by Van Maele-Fabry⁸, mouse embryos were explanted at E9.5. Using a high-magnification stereomicroscope and forceps to visualize and orient the embryos properly, 15 to 18 somite embryos with yolk sac and ectoplacental cone were selected and cultured in undiluted serum (80% adult human serum and 20% adult Sprague-Dawley rat serum) for 4h at 37 °C, the gas phase being 5% CO₂ and 95% O₂. The embryos were cultured in 0.5 ml medium per embryo in propylene tubes which were placed on a rotating agitator (30 r.p.m.). VIP (Peninsula), VA, VIP + VA, or secretin (Peninsula) were added to the culture medium: VIP was diluted in 1 μ l 0.02% acetic acid per ml culture medium; VA and secretin were diluted in 10 μ l PBS per ml culture medium. The culture medium was not modified during the culture period. The VIP concentrations were identical in the medium and the embryo within 5 minutes, and, after 2h, 20% of the VIP was apparently still intact (fast protein liquid chromatography data not shown). After 4h, the number of somites was counted under a stereomicroscope and the embryos were fixed for 2h in 4% formalin followed by dehydration and paraffin embedding. Sagittal 5 μ m serial sections were cut and stained with haematoxylin and eosin. The areas of section of the whole embryos were measured using an image analyser. These areas were compared at the level of the largest section of each embryo (section at the level of the heart left ventricle) to avoid regional variation.

FIG. 2 VIP promotes embryonic growth without inducing macroscopic abnormalities. (1) Typical appearance of the explanted embryos before the culture (T_0). Note that the yolk sac and ectoplacental cone were removed to allow a comparison with the cultured embryos. (2)–(5). These 4-h cultured embryos were processed in the same experiment and represent the typical macroscopic aspect observed in repeated cultures with 10^{-10} , 10^{-9} , or 10^{-7} M VIP and 10^{-7} M secretin (S) when compared with control cultured embryos (C). The explantation and culture were done as described in Fig. 1. At the end of the culture, the embryos were fixed for 2 h in formalin. Scale bar, 0.25 mm.



CNS while decreasing the same in non-neuronal tissues by 38%. *In vitro* autoradiography revealed GTP-sensitive and GTP-insensitive VIP receptors which were differentially regulated in VIP antagonist-treated embryos. The present study suggests that VIP acts as a growth factor on early postimplantation embryos through multiple VIP receptors that exhibit tissue-specific responses.

Controlled exposure of *in vitro* whole embryos of known somitic stage^{7,8} was used to circumvent two major problems inherent to the *in vivo* administration of a drug to a pregnant animal: a lack of precise staging of embryos and variation in the amount of drug exposure. Postimplantation NIH Swiss mouse embryos were explanted with their yolk sac at embryonic day (E) 9.5. Embryos, displaying 15 to 18 somites, were cultured *in toto* for 4h in the presence of various neuropeptides.

To assess the potential effect of VIP on whole embryo growth, the number of somites (a growth indicator¹¹) and the area of the largest sagittal section (estimate of embryo volume) were used. At E9–E10, *in vivo* embryos acquire one new somite every 1–2 hours¹². Here, as in previous *in vitro* studies^{7,8,13}, control embryos formed an average of 2.2 new somites during the culture period (Fig. 1a). VIP treatment produced a concentration-dependent increase in somitic number (an average of 3.9 and

5.2 new somites were produced with 10^{-9} and 10^{-7} M VIP, respectively), whereas addition of secretin or a VIP antagonist (VA) alone had no effect. Similar VIP growth-stimulating effects (11–63% of increase from control) were evident on embryonic volume (Fig. 1b). A 100-fold excess of VA partially blocked the VIP-stimulated increases in embryonic growth, suggesting receptor specificity (Fig. 1a, b). Both the macroscopic (Fig. 2) and microscopic (data not shown) examination of VIP-treated embryos revealed no apparent abnormalities. However, cotreatment with VIP and VA resulted in embryonic malformation, including head deformation and irregularity of the neuroepithelium (data not shown).

To discriminate between an increase in cell size versus cell number in VIP-treated embryos, DNA and protein content were measured. In comparison to control animals in culture, VIP increased the DNA and protein content by 103% and 63%, respectively (Fig. 3a). Coadministration of the VIP antagonist (100-fold excess) inhibited the VIP increases by about 75%. Addition of the antagonist or secretin alone had no effect. Because the DNA and protein content increased proportionally, cell division appears to be the major mechanism accounting for the VIP-induced enlargement of the embryos.

FIG. 3 VIP stimulates cell mitosis in the different tissues of whole cultured embryo. Bar represents mean $\pm$ s.e.m.; asterisks indicate difference from control (* P < 0.05, ** P < 0.01 or less in ANOVA); $\blacktriangle$, differs from the group treated with a similar concentration of VIP but without VA ($\blacktriangle$, P < 0.05, $\blacktriangle\blacktriangle$ P < 0.01 or less in ANOVA). a, VIP increases total DNA (■) and protein (▨) contents in a parallel way. Embryos were dissected and cultured as described in Fig. 1. In each group, a total of five embryos from three separate culture experiments were pooled and rapidly frozen at -80°C . After thawing, the embryos were homogenized in 0.1M perchloric acid. The DNA content was measured, according to the method described by Burton²⁸ and modified by Munro²⁹ and the protein content was measured using the Biorad protein assay³⁰. b, VIP increases the number of cells in S phase in neural and non-neural tissues. In each group, a total of 5–18 embryos from at least three separate experiments were included. In comparison, 10^{-6} M VIP doubled the incorporation of tritiated thymidine in cultured cells from rat embryonic superior cervical ganglia³¹. Embryos were explanted and cultured as described in Fig. 1 except that 1h before the end of the incubation, BrdU (Sigma) at a final concentration of 1 mM was added to the culture medium. This protocol was chosen because it is associated with labelling of 100% of nuclei in S phase in a proliferative neuroepithelium³². At termination, embryos were formalin-fixed, paraffin-embedded and sagittally serially cut as described in Fig. 1. Nuclei that have incorporated BrdU during the incubation period were detected by immunohistochemistry³². In each embryo, positive cells were counted in neural and non-neural tissues on five sections (taken at 10 μm intervals) at the level of the heart to avoid regional variation between embryos.

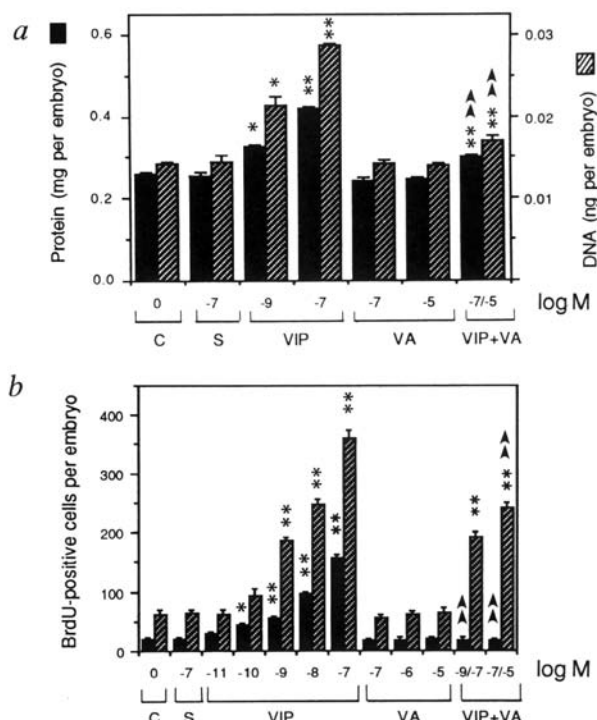
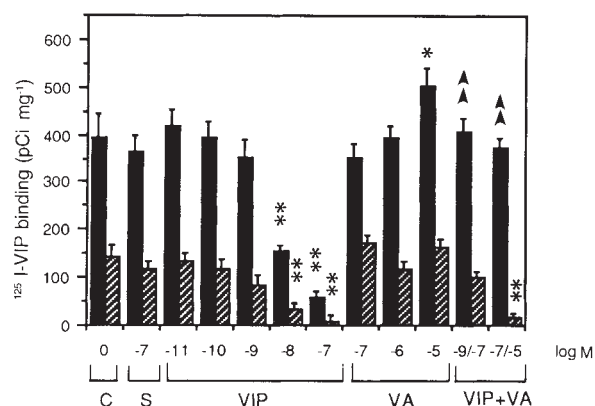


FIG. 4 VIP downregulates *in vitro* ^{125}I -VIP binding in whole cultured embryos. The effects on the GTP-insensitive (■) and GTP-sensitive (▨) receptors of different concentrations of VIP, VA, VIP+VA, or secretin (S) added to the culture medium are shown in comparison with the binding observed in control embryos (C) cultured with medium alone. In each group, a total of 5–15 embryos from four separate experiments were included. Bar represents mean $\pm$ s.e.m.; asterisks indicate difference from control (* $P < 0.05$, ** $P < 0.01$ or less in ANOVA); ▲, differs from the group treated with a similar concentration of VIP but without VA (▲, $P < 0.05$; ▲▲, $P < 0.01$ or less in ANOVA). The embryos were explanted and cultured as described in Fig. 1. At the end of the incubation period, embryos were extensively washed in PBS and rapidly frozen at -80°C . Cryostat sections (20 μm) were processed for binding with ^{125}I -VIP and for subsequent autoradiography as described by Hill *et al.*¹⁵. Sections were incubated in ^{125}I -VIP with and without 1 μM VIP (Peninsula) or 10 μM of a stable guanosine 5'-triphosphate analogue, guanyl-imidodiphosphate (Boehringer Mannheim). Two classes of VIP-binding site can be differentiated on the basis of their sensitivity to guanine nucleotides¹⁵. The GTP-sensitive binding site is probably the adenylate cyclase-linked VIP receptor which stimulates cyclic AMP pathways. Recent evidence suggests that the GTP-insensitive receptor may operate through a phospholipid signal transduction mechanism^{33,34}. The density of labelled VIP in various regions of the embryo neural tube was analysed by digitizing the film images using a Macintosh-II-based image analysis system (IMAGE.



Wayne Rasband, Research Services Branch, NIMH). Specific binding was determined by subtracting the light transmittance from brain sections incubated with 1 μM unlabelled VIP from the total light transmittance. The relative amount of ^{125}I -VIP specifically bound was converted to picocuries (pCi) per mg wet weight of tissue using the standard curve generated by ^{125}I -standards (Amersham).

To measure directly the effect of VIP treatment on mitosis and to determine the extent to which neural and/or non-neural tissues were involved in the VIP-related growth processes, bromodeoxyuridine (BrdU), a thymidine analogue that is integrated in dividing cells at the S-phase¹⁴, was added to the culture medium 1 hour before the termination of the experiment. VIP, but not secretin or VA alone, produced a concentration-dependent increase in the number of cells in S-phase (Fig. 3b). A 5–6-fold increase in mitosis was elicited by VIP treatment in both neural and non-neural tissues. When embryos were treated with a combination of VIP and VA, the VIP-induced increase in BrdU-positive cells in the CNS was completely prevented by the VIP antagonist; whereas in non-neural tissues, the antagonist blocked a maximum of 38% of the increase (Fig. 3b). The tissue-specific effect of the VIP antagonist on BrdU-positive cells implies that regulation of mitosis occurred through pharmacologically distinct VIP receptors. The abnormal morphology observed with VIP and VA cotreatment may be related to disproportionate growth secondary to the differential selectivity of VA for receptors regulating the CNS versus non-CNS effects.

As shown in rat embryos^{1,2}, most of the specific binding of ^{125}I -VIP in the mouse embryo explant was restricted to the CNS, including spinal cord and cephalic vesicles. Non-neural tissue displayed few VIP-binding sites (data not shown). The VIP-binding sites were differentiated into two subtypes in the brain, based on their sensitivity to guanine nucleotides¹⁵ (Fig. 4). In control embryos in culture, most (73%) of the VIP binding was GTP-insensitive, similar to that reported for rat embryos^{1,2}. Addition of VIP to the culture medium produced a concentration-dependent decrease in receptor density at both GTP-sensitive (42–92%) and GTP-insensitive (11–85%) sites. In contrast, VA produced an increase (27%) in the density of GTP-insensitive binding sites, but had no apparent effect on GTP-sensitive sites. Specificity was demonstrated in that secretin treatment of whole embryos had no effect on VIP binding. Cotreatment of embryos with VIP and VA resulted in a 93–100% block of the VIP-related reduction in GTP-insensitive binding sites but had no effect on GTP-sensitive sites. On the basis of the differential effect of VA on nucleotide-regulated VIP binding sites, GTP-insensitive sites appear to mediate the growth-promoting actions of VIP in the CNS.

Because most of the VIP receptors were localized in the CNS, the mechanism by which VIP stimulates non-neural tissue growth is unclear. Two pharmacologically distinct receptors mediate the VIP growth responses and thus two alternative mechanisms are feasible: (1) that there are tissue-specific VIP

receptors that mediate the regional responses; here a direct action of VIP on the few non-CNS receptors could result in growth because VIP stimulates mitosis of cultured non-neural cells^{16–18}; (2) that there are two pharmacologically distinct VIP receptors in the CNS, one that mediates effects on CNS cells and another that acts indirectly by influencing the non-CNS responses through secondary molecules. In the latter context, stimulation of cultured astrocytes with VIP results in the release of trophic substances^{19,20}. An analogous mechanism could occur in cultured embryos, with glial CNS cells¹³ releasing substances that diffuse to influence non-CNS cells.

VIP seems to be a crucial determinant of embryonic growth. Although the dramatic growth spurt after treatment with high (0.1 μM) concentrations of VIP is probably pharmacological in terms of magnitude and kinetics, two observations suggest a physiological role in ontogenesis: (1) VIP has a neurotrophic action on embryonic cells grown *in vitro*^{3,4}; (2) there are high levels of VIP-binding sites in the CNS throughout development^{1,2}. *In utero*, the regulation of growth may be mediated by a more prolonged exposure to lower concentrations of VIP. The present conditions of culture do not permit longer incubation times than used here without inducing malformations in control embryos^{8,21}. VIP messenger RNA levels^{1,22} and VIP immunoreactivity^{23,24} are low in the rat embryo, especially in the brain, suggesting that *in vivo* regulation of prenatal neural development may occur either by extraembryonic (maternal and/or placental) VIP or by a related molecule such as pituitary adenylate cyclase-activating peptide. This peptide has been shown to act potently on some VIP receptors²⁵ but its production in embryonic tissues has not yet been reported. Apparently, no other growth factors exhibit the growth-stimulating effects of the magnitude demonstrated here for VIP, although insulin has been reported to stimulate the incorporation of tritiated thymidine (8–27%) in cultured preimplantation mouse embryos²⁶.

VIP has a dramatic effect on *in vitro* embryonic growth that seems to be mediated through multiple, tissue-specific mechanisms involving at least two pharmacologically distinct VIP receptors. These data imply that VIP has a vital endocrine role in the regulation of embryonic development. □

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Selection is not required to produce invariant T-cell receptor γ -gene junctional sequences

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RECOMBINATION of V-, D- and J-gene segments can generate an enormous diversity of T-cell antigen receptor (TCR) gene sequences^{1,2}. Although many $\gamma\delta$ T cells fully exploit this diversification process, those in the epidermal and vaginal epithelium do not^{3,4}, predominantly expressing invariant $\gamma\delta$ receptors in which the V-(D)-J junctional sequences in almost all the productive rearrangements are identical. The almost exclusive use of identical TCRs by cells in these sites is thought to reflect recognition of a stress-induced autologous antigen^{5–8}. To explain the prevalence of the invariant junctional sequences, it has been proposed that thymic selection operates on a population of originally diverse progenitor cells, resulting in a homogeneous repertoire^{9,10}. Alternatively the invariant sequences may result from biases in the recombination machinery in the fetal thymic progenitors of these cells^{8,11,12}. We report here the use of mice into which mutated TCR γ -gene rearrangement substrates have been introduced as transgenes to demonstrate directly that the canonical TCR $V\gamma 3$ - $J\gamma 1$ and $V\gamma 4$ - $J\gamma 1$ sequences occur at high frequency in the absence of the possibility of selection for the protein products.

We assembled a transgene construct in which the unrearranged $V\gamma 2$, $V\gamma 4$ and $V\gamma 3$ gene segments¹³ were placed upstream of a fragment containing the unrearranged $J\gamma 1$ - $C\gamma 1$ gene segment and the $C\gamma 1$ enhancer element^{13–15} (Fig. 1a). Frameshift mutations were introduced into the coding regions of each of the $V\gamma$ genes, producing downstream termination codons before the 3' end of each $V\gamma$ coding sequence (see Fig.

1 legend). Hence rearrangement of the transgenes will be independent of any selection acting on the cells.

The transgenes were introduced into (C57BL/6 $\times$ CBA/J) F_2 fertilized eggs, and transgenic founder animals were identified by Southern blot analysis of tail DNA. One transgenic founder female was crossed to a CBA/J male. Genomic thymocyte DNA was prepared from seven newborn pups, of which three were identified as transgenic. With the use of primers specific for linker sequences that had been introduced into the $V\gamma 3$ and $V\gamma 4$ transgenes, in conjunction with a downstream $J\gamma 1$ primer, rearrangements of the transgenic substrates could be specifically amplified (see Fig. 1 legend). $V\gamma 3$ - $J\gamma 1$ and $V\gamma 4$ - $J\gamma 1$ transgene rearrangements were both detected in the newborn thymocytes (Fig. 1b, lanes 3–5 and 9–11). Using the same primer pairs, no polymerase chain reaction (PCR) products were obtained with genomic DNA from a nontransgenic littermate, indicating that these primers fail to amplify the endogenous, wild-type rearrangements.

To examine the junctional sequences of the rearranged transgenes, the $V\gamma 3$ - $J\gamma 1$ PCR products from the thymocytes of the newborn mice were cloned and examples were sequenced. The 5' ends of all the sequences contained, in the expected location, the upstream restriction enzyme linker that was used to anchor the transgene-specific primer, confirming their origin from the mutant transgene substrates (data not shown). Of 17 $V\gamma 3$ - $J\gamma 1$ rearrangements that generated an in-frame junctional sequence, 14 (82%) corresponded to the $V\gamma 3$ - $J\gamma 1$ canonical junctional sequence previously described^{3,5,9} (Table 1). This percentage is nearly as high as that reported for $V\gamma 3$ - $J\gamma 1$ rearrangements from thymocytes of normal newborn and fetal mice⁹, where 26 of 27 in-frame sequences (96%) were of the canonical type. Our preliminary analysis of $V\gamma 4$ - $J\gamma 1$ rearrangements from the transgenic mice indicates that the canonical junctional sequence^{4,16} occurs in two of three in-frame examples (Table 1). These results establish that the canonical γ -gene rearrangements occur at a very high frequency in the absence of selection for the products of the rearrangements.

The paucity of N-region nucleotides in these rearrangements clearly contributes to limiting their junctional diversity⁵. But even in the absence of N-region addition, 17 or more other in-frame sequences are possible, depending on how deeply the recombinase can penetrate into the $V\gamma$ and $J\gamma$ gene segments. Only eight of these have been observed, and each is very rare (Table 2). The canonical $V\gamma 3$ - $J\gamma 1$ rearrangement, unlike the other observed in-frame rearrangements, occurs at the site of a two-base-pair (bp) direct repeat (AT, see Table 1). Therefore, as suggested elsewhere^{11,12,17–19}, the tendency of V-(D)-J recombination to occur at the sites of direct repeats in the recombining gene segments may contribute importantly to the prevalence of the canonical rearrangements.

The 35 other $V\gamma 3$ - $J\gamma 1$ transgene sequences (67.3%) were joined in the incorrect translational reading frame. This is a higher percentage than reported for the rearrangement of the endogenous $V\gamma 3$ - $J\gamma 1$ genes in the thymus of fetal and newborn normal mice, where 18 of 45 (40%) of the rearrangements were nonproductive⁹. It is likely that the higher frequency of productive rearrangements among the normal versus transgene rearrangements is due to the selective survival and accumulation of cells expressing productively rearranged TCR genes that can encode a cell-surface TCR.

Several of the out-of-frame rearrangements occur at a relatively high frequency in the transgenic rearrangements (Table 1), as well as in the out-of-frame $V\gamma 3$ - $J\gamma 1$ rearrangements reported in the literature (Table 2). The addition of P (palindromic) elements⁹ to the ends of the V or J segments before recombination can create direct repeats that may in some cases account for the high frequency of the corresponding out-of-frame rearrangement. For example, the relatively frequent rearrangements E and F occur at the sites of 2–3-bp direct repeats created by P-element addition (Tables 1, 2). Finally, it should be noted

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TABLE 1 Junctional sequences of rearranged transgenic *V γ 3-J γ 1* and *V γ 4-J γ 1* gene segments

<i>Vγ3</i> <i>Jγ1</i>		GCC TGC TGG GAT CT CACAGTG				CACTGTG AT AGC TCA GGT			
Type	Frequency*	<i>Vγ3</i>	P	N	P	<i>Jγ1</i>	In frame†	Rpt‡	
A	14	GCC TGC TGG GA				T AGC TCA GGT	+	AT	
B	1	GCC TGC TGG GA				T	+	T	
C	1	GCC TGC TGG GA			AT	AT AGC TCA GGT	+	—	
D	1	GCC TGC TGG GAT C				AT AGC TCA GGT	+	—	
E	9	GCC TGC TGG GAT CT				AGC TCA GGT	—	TAG§	
F	6	GCC TGC TGG GAT				AT AGC TCA GGT	—	AT§	
G	3	GCC TGC TGG G			T	AT AGC TCA GGT	—	—	
H	2	GCC TGC TG				AT AGC TCA GGT	—	—	
I	2	GCC TGC TGG GAT CT				AT AGC TCA GGT	—	TA§	
J	1	GCC TGC T			AT	AT AGC TCA GGT	—	—	
K	1	GCC TGC TGG G				T AGC TCA GGC	—	—	
L	1	GCC TGC TGG GAT C				AGC TCA GGT	—	—	
M	1	GCC TGC TGG GAT C				AGC TCG GGT	—	—	
N	1	GCC TGC TGG GAT				T AGC TCA GGT	—	—	
O	1	GCC TGC TGG				AT AGC TCA GGT	—	—	
P	1	GCC TGC TGG			AT	AT AGC TCA GGT	—	—	
Q	1	GCC TGC TGG GAT CT	AG			T AGC TCA GGT	—	—	
R	1	GCC TGC TGG GA		CA		AGC TCA GGT	—	—	
S	1	GCC TGC TGG GA		AGGGGG		AGC TCA GGT	—	—	
T	1	GCC TGC TGG GAT		AA		AGC TCA GGT	—	—	
U	1	GCC TGC TGG G		G		AGC TCA GGT	—	—	
V	1	GCC TGC TGG GAT C		GAGG		AGC TCA GGT	—	—	
<i>Vγ4</i> <i>Jγ1</i>		GCA TGC TGG GAT A CACAGTG				CACTGTG AT AGC TCA GGT			
Type	Frequency	<i>Vγ4</i>	P	N	P	<i>Jγ1</i>	In frame	Rpt	
AA	2	GCA TGC TGG GA				T AGC TCA GGT	+	AT	
BB	1	GCA TGC TGG GA			AT	AT AGC TCA GGT	+	—	
CC	1	GCA TGC TGG GA				AGC TCA GGT	—	—	
DD	1	GCA TGC TGG GAT				AT AGC TCA GGT	—	TAT§	
EE	1	GCA TGC TGG		A	AT	AT AGC TCA GGT	—	—	

The 3' end of the germ line *V γ 3* or *V γ 4* and 5' end of the germ line *J γ 1* gene segments are shown with their recombination signal heptamer sequences. Sequences 'A' and 'AA' are the canonical *V γ 3* and *V γ 4* sequences, respectively, found at high frequency in other studies^{4,5,10,19}. N and P refer to N-region and P-element additions.

* The number of times the identical sequence was found.

† The sequences are designated plus or minus to indicate whether the appropriate reading frame of the *V* and *J* gene segments is maintained.

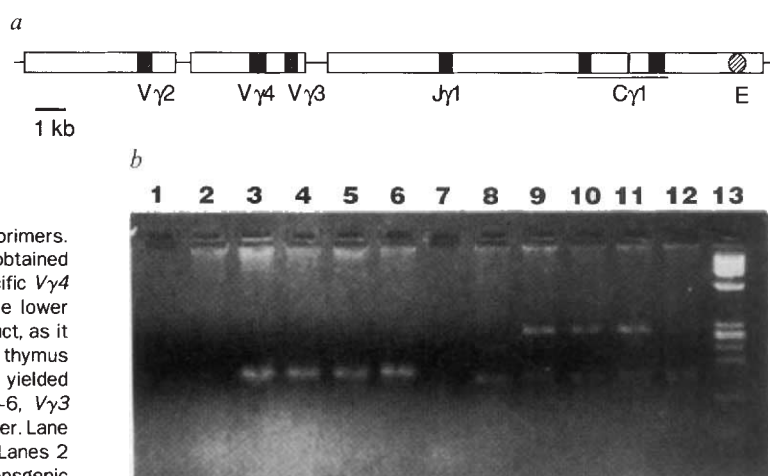
‡ The direct repeat of nucleotides, if any, found at the recombination breakpoints of the *V* and *J* gene segments.

§ In these cases, the direct repeats are present only after P-element addition.

|| These sequences include a single base change 3' of the junction in the *J γ 1* sequence, possibly due to errors by *Taq* polymerase.

FIG. 1 TCR γ -gene construct undergoes V-J rearrangement in transgenic mouse newborn thymus DNA. **a**, Structure of the rearrangement substrate construct. Shaded boxes represent exons; open boxes, introns; stippled boxes, introduced linkers (not to scale); thin lines, polylinker sequences (not to scale); and hatched oval, the *C γ 1* enhancer. **b**, Rearrangement of the transgenic *V γ 3* and *V γ 4* genes in newborn thymus DNA was detected by PCR using transgene-specific *V γ* primers and a *J γ 1* primer. No specific products were obtained from a nontransgenic littermate with either the *V γ 3* (lane 2) or *V γ 4* (lane 8) upstream primers. Transgene-specific *V γ 3* products of the expected 162 bp size were obtained from 3 transgenic littermates (lanes 3–5), as were transgene-specific *V γ 4* products of the expected 279 bp size (top band, lanes 9–11). The lower band in lanes 9–11 appears to be a nonspecific amplification product, as it is also obtained from non-*V γ 4* transgenic DNA (lanes 8, 12). Newborn thymus DNA from a transgenic mouse produced with a *V γ 3*-only construct yielded a specific product only with the *V γ 3* primer (lane 6). Lanes 1–6, *V γ 3* transgene-specific primer. Lanes 7–12, *V γ 4* transgene-specific primer. Lane 13, Φ X174-Hae III digest as marker. Lanes 1 and 7, no template. Lanes 2 and 8, nontransgenic littermate. Lanes 3–5 and 9–11, littermates transgenic for the *V γ 2/V γ 4/V γ 3 construct. Lanes 6 and 12, newborn thymus transgenic for a *V γ 3*-only construct.*

METHODS. The construct was assembled from separate genomic clones containing *V γ 2*, *V γ 4* and *V γ 3*, and *J γ 1*, *C γ 1*, and the *C γ 1* enhancer. About 3 kilobases (kb) of the genomic DNA between *V γ 2* and *V γ 4*, and about 5 kb of the genomic DNA between *V γ 3* and *J γ 1*, are included. The *V γ 2* gene was cut with *Clal*, blunted, and a 12-bp *XhoI* linker was inserted. The *V γ 4* gene was cut with *KpnI*, blunted, and an 8-bp *XhoI* linker was inserted. The resulting frameshifts produce termination codons 2 and 13 codons downstream for the *V γ 2* and *V γ 4* genes, respectively. The *V γ 3* gene was digested at the 3' *KpnI* site, blunted, and a 10-bp *EcoRI* linker was inserted. The gene was then cut at the 5' *KpnI* site, blunted, and religated. This produced a termination codon 2 codons downstream. PCR was done on genomic DNA isolated



from newborn thymuses, and one-tenth of the 100- μ l reactions were electrophoresed on a 3% Nusieve/1% Seakem agarose gel. Control experiments were done in which PCR (using the identical conditions) of a mixture of transgenic tail DNA and normal newborn thymus DNA failed to yield any detectable products (data not shown). This ruled out the possibility that the observed products were the result of overlap extension during the PCR of unrearranged transgene-derived DNA strands and strands from rearranged endogenous genes. The *V γ 3* primer was GATGAGAAGGATGATGCGGAA. The *V γ 4* primer was GATGCATACATCACTGCCTC. The last 5 bases of the *V γ 3* primer and the last 4 bases of the *V γ 4* primer are found in the introduced linkers but not the endogenous genes. The *J γ 1* primer was GCGAAGCTTCAGAGGGAATTACTATGA.

TABLE 2 Compilation of *V γ 3-J γ 1* junctional sequences

Type	<i>Vγ3</i>	P	P	<i>Jγ1</i>	Number observed*	Rpt†
In frame						
A	GCC TGC TGG GA			T AGC TCA GGT	109	2
	GCC TGC T			AT AGC TCA GGT	2	1‡
B	GCC TGC TGG GA			T	1	1
	GCC TGC TGG GAT CT	AG		AT AGC TCA GGT	1	1‡
	GCC TGC TGG GAT CT	A		AGC TCA GGT	1	0
	GCC TGC TGG GAT C			GC TCA GGT	1	0
	GCC T			AT AGC TCA GGT	1	1‡
C	GCC TGC TGG GA		AT	AT AGC TCA GGT	1	0
D	GCC TGC TGG GAT C			AT AGC TCA GGT	1	0
Out of frame						
E	GCC TGC TGG GAT CT			AGC TCA GGT	16	3‡
F	GCC TGC TGG GAT			AT AGC TCA GGT	13	2‡
I	GCC TGC TGG GAT CT			AT AGC TCA GGT	4	2‡§
G	GCC TGC TGG G		T	AT AGC TCA GGT	3	0
N	GCC TGC TGG GAT			T AGC TCA GGT	2	0
O	GCC TGC TGG			AT AGC TCA GGT	2	0
H	GCC TGC TG			AT AGC TCA GGT	2	0
	GCC TGC TGG GAT C		AT	AT AGC TCA GGT	2	0
	GCC TGC TGG GAT		AT	AT AGC TCA GGT	2	1‡
K	GCC TGC TGG G			T AGC TCA GGT	2	0
P	GCC TGC TGG		AT	AT AGC TCA GGT	2	0
	GCC TGC TGG GAT CT		T	AT AGC TCA GGT	1	0
L	GCC TGC TGG GAT C			AGC TCA GGT	2	0
Q	GCC TGC TGG GAT CT	AG		T AGC TCA GGT	1	0
	GCC TGC TGG G			AGC TCA GGT	1	1
	GCC			T AGC TCA GGT	1	1
	GCC TGC			AT AGC TCA GGT	1	0

A summary of the junctional sequences of *V γ 3-J γ 1* rearrangements from the literature. Only sequences that lack N-nucleotide addition are included. Sequences from refs 13, 21, 5, 3, 9, 10 and Table 1 of the present paper are included. The letters to the left identify sequences that are also listed in Table 1.

* The total number of reported examples of the corresponding sequence, from the references listed.

† The length of direct repeats, if any, shared by the *V γ 3* and *J γ 1* sequences and that correspond to the crossover point in the indicated rearrangement.

‡ In these cases the direct repeats are present only after addition of P elements of 1–3 bp.

§ In this case P-element addition to both the *V* and *J* segments is required to generate the 2-bp direct repeat. This may explain the lower than expected frequency of this rearrangement.

|| In each of these cases, two sequences have been grouped together, where one of the sequences includes a single base pair change in *J γ 1* 3' of the junction (see Table 1).

that the junctions of the canonical *V δ 1-D δ 2-J δ 2* rearrangement⁵ also occur at the sites of direct repeats; the 2-bp direct repeat to generate the *D δ 2-J δ 2* junction corresponds to a 3-bp P-element addition to the end of the *J δ 2* gene segment. Apart from the role of direct repeats in aligning the recombining gene segments, the recombinase machinery may play some other unknown role in directing the production of the canonical rearrangements.

Two arguments have been advanced in favour of the model that the invariant junctional sequences predominate as a result of selection. First, Lafaille *et al.*⁹ found that the junctions of out-of-frame rearrangements were more diverse than the in-frame (predominantly canonical) sequences and suggested that selection of the protein products of the productive rearrangements had occurred. But this argument assumes that rearrangement is random, contrary to the observations that rearrangements tend to occur at the sites of direct repeats. Second, Itohara *et al.*¹⁰ found that the diversity of in-frame rearrangements was increased in fetal thymus organ cultures treated with anti- $\gamma\delta$ TCR antibodies, and suggested that such treatment had interfered with putative selective processes. But such effects were not observed in a similar analysis using anti- $\gamma\delta$ TCR antibodies in fetal thymic organ culture (A. Carbone, J. Noble and J. P.

Allison, personal communication). Nevertheless, our data do not directly address the possibility that selection acts after directed rearrangement to promote the survival of cells with the invariant receptors.

Our results indicate that the production of the invariant junctional sequences of the TCR γ -chains used by the epithelial-associated $\gamma\delta$ T cells of the skin and reproductive tissues is a highly directed process that is imposed by the recombination machinery. Evidence is accumulating that the use of specific *V γ* gene segments by cells in these populations is also regulated at the level of rearrangement^{12,20}. Apparently, the task of producing populations of cells with such highly restricted specificity can be accomplished by directing the types of rearrangements that occur, without requiring the selection of rare cells with this specificity from a heterogeneous population. □

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Mutations in CFTR associated with mild-disease-form Cl^- channels with altered pore properties

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THE cystic fibrosis transmembrane conductance regulator (CFTR) is a phosphorylation-regulated Cl^- channel located in the apical membrane of epithelia^{1–10}. Although cystic fibrosis (CF) is caused by mutations in a single gene encoding CFTR^{11,12}, the disease has a variable clinical phenotype^{13,14}. The most common mutation associated with cystic fibrosis, deletion of a phenylalanine at position 508 (frequency, 67%), is associated with severe disease^{15–17}. But some missense mutations, for example ones in which arginine is replaced by histidine at residue 117 (R117H; 0.8%), tryptophan at 334 (0.4%), or proline at 347 (0.5%), are associated with milder disease^{15,17,18}. These missense mutations affect basic residues located at the external end of the second (M2) and in the sixth (M6) putative membrane-spanning sequences. Here we report

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that, when expressed in heterologous epithelial cells, all three mutants were correctly processed and generated cyclic AMP-regulated apical Cl^- currents. Although the macroscopic current properties were normal, the amount of current was reduced. Patch-clamp analysis revealed that all three mutants had reduced single-channel conductances. In addition, R117H showed altered sensitivity to external pH and had altered single-channel kinetics. These results explain the quantitative decrease in macroscopic Cl^- current, and suggest that R117, R334 and R347 contribute to the pore of the CFTR Cl^- channel. Our results also suggest why R117H, R334W and R347P produce less severe clinical disease and have implications for our understanding of cystic fibrosis.

Many CF-associated mutations, including the most common ΔF508 , cause a defect in protein processing and a failure of mutant CFTR to reach the apical membrane^{6,19–21}. To assess the processing and function of R117H, R334W, and R347P, we expressed wild-type and mutant CFTRs in Fischer rat thyroid (FRT) epithelial cells^{1,22,23}. We chose these cells because they form polarized epithelia with a transepithelial resistance when grown on permeable filter supports and they have no cAMP-regulated Cl^- channels in their apical membranes (our unpublished results). Expression of wild-type CFTR and all three mutants generated the mature, fully glycosylated form of the protein (band C)^{19,24}, consistent with delivery to the plasma

membrane (Fig. 1a). In contrast, ΔF508 produced only the immature, core-glycosylated form of the protein (band B), a result indicative of defective processing^{19–21}. Immunofluorescence studies of unpermeabilized FRT epithelia stained with an antibody against an extracellular epitope also indicated that R117H, R334W and R347P were delivered to the plasma membrane (data not shown).

To assess the function of these 'mild' mutants, we permeabilized the basolateral membrane and measured cAMP-stimulated current flowing through apical membrane CFTR Cl^- channels (ref. 25, and our unpublished results). Figure 1b shows that cAMP agonists stimulated apical Cl^- current in epithelia expressing wild-type CFTR and each of the mutants associated with mild disease, but not in epithelia expressing ΔF508 . Although R117H, R334W and R347P retained significant apical membrane Cl^- channel function, the magnitude of current was reduced, with wild-type CFTR > R347P > R117H > R334W > ΔF508 .

To determine why Cl^- current was reduced, we studied the mutants using the whole-cell patch-clamp technique. Figure 2a summarizes the whole-cell properties of R117H; similar results were obtained with R334W and R347P (data not shown). Whole-cell Cl^- currents were reversibly activated by cAMP agonists, were time-independent, had linear current-voltage ($I-V$)

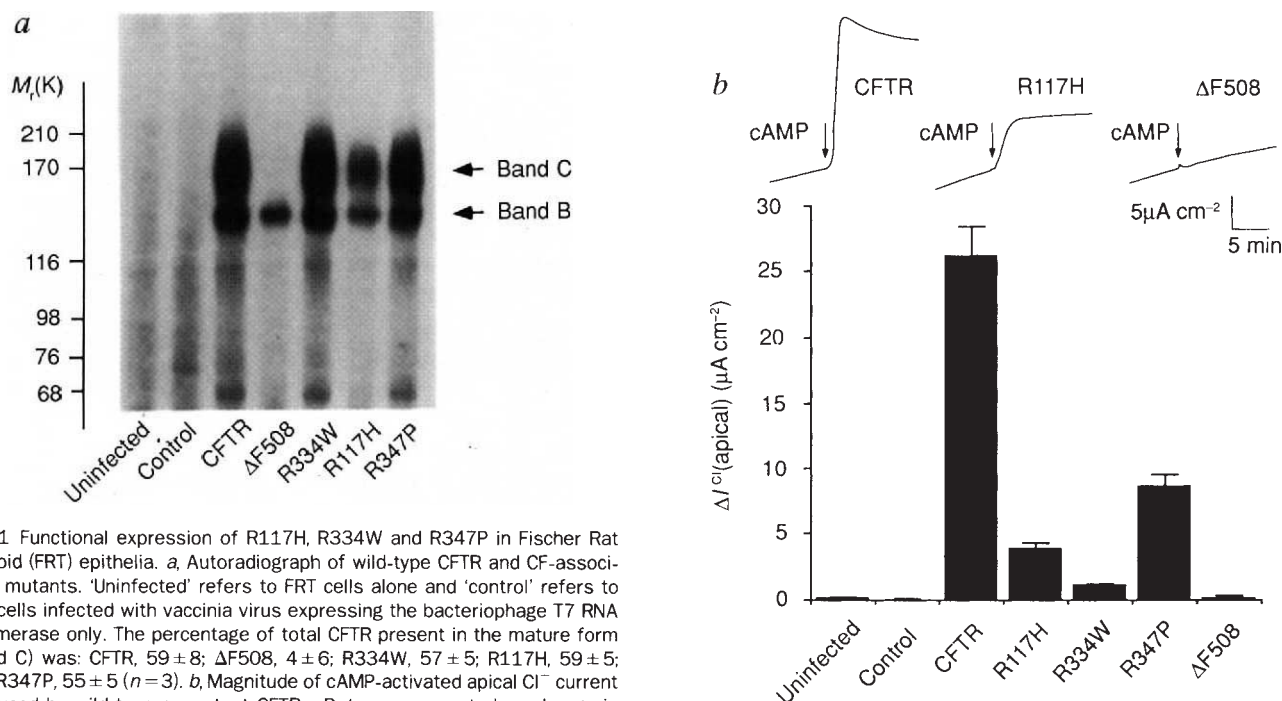


FIG. 1 Functional expression of R117H, R334W and R347P in Fischer Rat Thyroid (FRT) epithelia. **a**, Autoradiograph of wild-type CFTR and CF-associated mutants. 'Uninfected' refers to FRT cells alone and 'control' refers to FRT cells infected with vaccinia virus expressing the bacteriophage T7 RNA polymerase only. The percentage of total CFTR present in the mature form (band C) was: CFTR, 59 ± 8 ; ΔF508 , 4 ± 6 ; R334W, 57 ± 5 ; R117H, 59 ± 5 ; and R347P, 55 ± 5 ($n=3$). **b**, Magnitude of cAMP-activated apical Cl^- current produced by wild-type or mutant CFTRs. Data are presented as change in current [$\Delta I^0(\text{apical})$] measured 2 min after addition of cAMP agonists ($10 \mu\text{M}$ forskolin and $100 \mu\text{M}$ IBMX). FRT epithelia were infected with identical concentrations of recombinant viruses (multiplicity of infection, $\text{MOI}=5$). Results are mean $\pm$ s.e.m. of $n=5$ observations, except for control, in which $n=4$. Inset shows representative recordings of apical Cl^- current; arrows indicate point of addition of cAMP agonists.

METHODS. FRT cells were seeded onto Millicell HA filters at density of 5×10^5 cells per cm^2 . Epithelia developed a high transepithelial electrical resistance, ($3,000 \pm 200 \Omega \text{ cm}^2$; $n=34$) 5 days after seeding. R117H, R334W and R347P were constructed using the CFTR plasmid pTM-CFTR4 as described¹⁹. Mutations are named using their single-letter amino acid code. We transiently expressed either wild-type CFTR or mutants in confluent FRT epithelia using the vaccinia virus-T7 hybrid expression system^{1,23}. We used a double-infection protocol with one recombinant virus expressing the bacteriophage T7 RNA polymerase and a second recombinant virus containing either wild-type or mutant CFTR¹⁰. In *a*, soluble lysates of FRT cells infected with identical concentrations of recombinant viruses ($\text{MOI}=5$) were immunoprecipitated with antibody M 1–4 (ref. 6), phosphorylated with [$\gamma\text{-}^{32}\text{P}$]ATP and the catalytic subunit of PKA²⁴ and separated on a 6%

SDS-polyacrylamide gel. Radioactivity was quantified using a radioanalytical imaging system (AMBIS, San Diego)²¹. For Fig. 1b, FRT epithelia were mounted in modified Ussing chambers 20–30 h after infection. The mucosal surface of the epithelium was bathed in a physiological solution containing (in mM): 135 NaCl, 1.2 CaCl_2 , 1.2 MgCl_2 , 2.4 K_2HPO_4 , 0.6 KH_2PO_4 , 10 HEPES, 10 dextrose, (titrated to pH 7.4 with NaOH). The composition of the submucosal solution was similar to the mucosal solution, except that 135 mM sodium gluconate replaced 135 mM NaCl, to give a Cl^- concentration of 4.8 mM. Solutions were maintained at 37°C and bubbled with O_2 . Apical Cl^- current ($I^0(\text{apical})$) was measured as described²⁵. Nystatin (0.36 mg ml^{-1}) was added to the submucosal solution to permeabilize the basolateral membrane. Transepithelial voltage (referenced to the mucosal solution) was clamped at 0 mV, and apical Cl^- current was recorded continuously. When the basolateral membrane is permeabilized in the presence of a transepithelial Cl^- concentration gradient, cAMP-stimulated current results from Cl^- flow through apical membrane CFTR Cl^- channels (ref. 25, and our unpublished results). Flow of Cl^- from the mucosal to the submucosal solution is shown as an upward current deflection.

relationships, and were selective for anions over cations. These properties resemble those of wild-type CFTR¹⁻³. We previously found that mutation of two other basic amino acids, lysine to glutamic acid within M1 and M6 (K95E and K335E³), altered the anion selectivity sequence from that of wild type. The anion selectivity of R117H, R334W and R347P, however, was unchanged (Fig. 2b).

Nevertheless, the whole-cell properties of these mutants differed from wild-type CFTR in two ways. First, the magnitude of cAMP-activated whole-cell Cl⁻ current was reduced. Although quantitation of whole-cell current is less accurate than that of apical membrane current because of cell-to-cell variability, the relative current amplitude followed the same sequence: wild-type CFTR > R347P > R117H > R334W (see Fig. 2 legend for values). Second, R117 is predicted to lie just at the extracel-

lular surface of M2. Because in R117H an amino acid with a charged group having a pK near neutrality is substituted for a strongly basic one, we considered that this mutation might alter the sensitivity of CFTR to external pH. Figure 2c shows that external pH had no effect on whole-cell currents of wild-type CFTR, but at pH 8.8 R117H Cl⁻ currents were significantly reduced; this effect was reversible. At acidic pH, histidine probably has a charge more like arginine, whereas at basic pH it is likely to have a negative charge. These results suggest that R117 may lie at the extracellular mouth of the channel where it senses external pH, thereby influencing the conduction pathway.

To understand why R117H, R334W and R347P generated less Cl⁻ current, we examined their single-channel properties using excised, inside-out membrane patches. Figure 3 shows that the mutants were reversibly activated by phosphorylation with

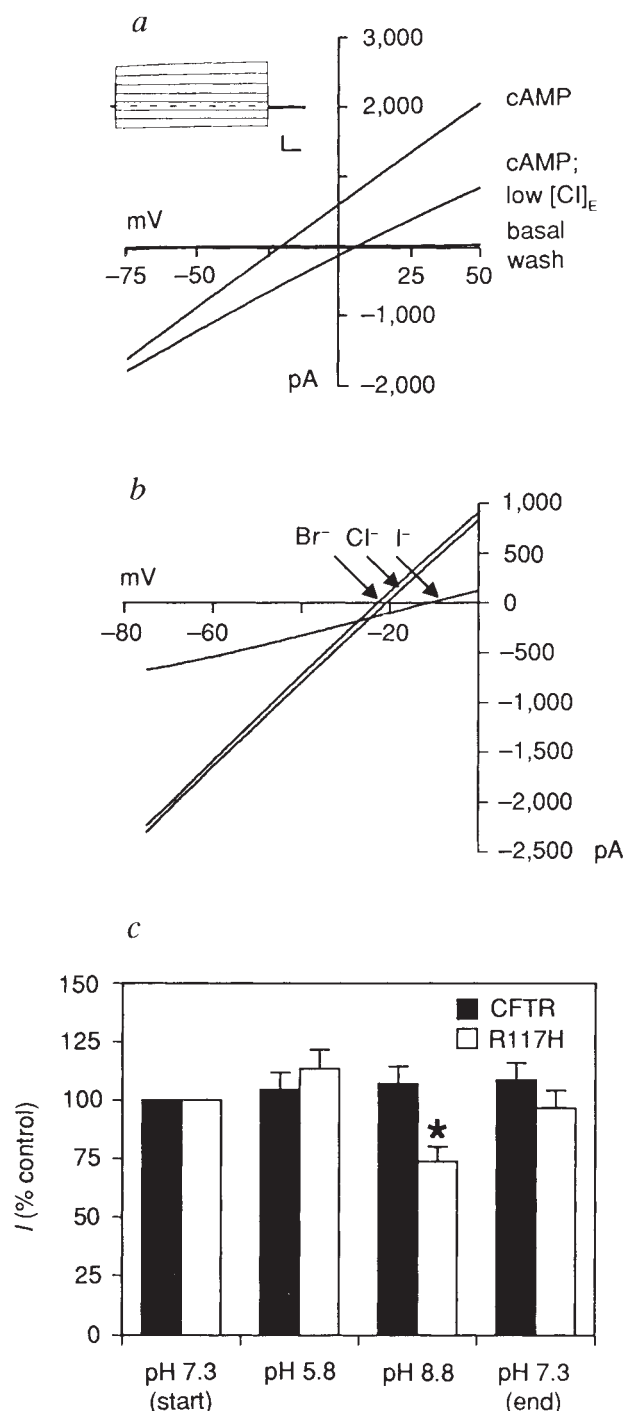


FIG. 2 Whole-cell properties of R117H. **a**, Current-voltage (*I-V*) relationships of whole-cell currents under basal conditions, 2 min after addition of cAMP agonists (10 μ M forskolin, 100 μ M IBMX, and 500 μ M 8-(4-chlorophenylthio)-adenosine-3',5'-cyclic monophosphate, sodium salt) to the bath solution and after removing cAMP (wash); $E_{rev} = -20$ mV ($n=8$); $E_{Cl} = -32$ mV ($P_{Na}/P_{Cl} = 0.17 \pm 0.0$; $n=8$). *I-V* relationships under basal and wash conditions are largely hidden under the abscissa 'cAMP; low [Cl]_E' indicates *I-V* relationship when bath Cl⁻ concentration was reduced to 20 mM (NaCl replaced with sodium aspartate) in the presence of cAMP agonists. *I-V* relationships were obtained by a 400-ms ramp of voltage; holding voltage was 0 mV. Inset shows a representative example of cAMP-activated whole-cell R117H Cl⁻ currents. Holding voltage was -30 mV, and voltage was stepped from -80 mV to +60 mV in 20 mV increments. Before cAMP agonists were added, steady-state current was -35 pA at -80 mV, and 113 pA at +60 mV. The dashed line represents the zero current level. Scale bars are 1,000 pA and 50 ms, respectively. cAMP-activated whole-cell Cl⁻ currents measured at +50 mV were: wild-type CFTR, 205.1 ± 28.1 pA/pF ($n=15$); R347P, 86.6 ± 20.4 pA/pF ($n=13$); R117H, 68.4 ± 11.5 pA/pF ($n=15$); and R334W, 10.3 ± 2.5 pA/pF ($n=15$); cell capacitance was 41.7 ± 1.7 pF ($n=58$). **b**, *I-V* relationships of cAMP-activated whole-cell currents in the presence of 140 mM Cl⁻, Br⁻ or I⁻ in the bath solution. *I-V* relationships were generated as described in **a**. Permeability ratios, P_X/P_{Cl} , were I⁻, 0.83 ± 0.04 ; Br⁻, 1.15 ± 0.05 , and conductance ratios, G_X/G_{Cl} , were I⁻, 0.39 ± 0.07 ; Br⁻, 1.26 ± 0.21 ($n=5$); values were calculated as described³. **c**, External alkaline pH inhibits R117H. Cyclic AMP-activated whole-cell currents were measured at +50 mV. Values are normalized to those measured at pH 7.3 at the start of the experiment. Results are mean $\pm$ s.e.m.; $n=6$ (CFTR) and 8 (R117H). Asterisk indicates a statistically significant difference ($P=0.002$). Inhibition of R117H whole-cell Cl⁻ currents at pH 8.8 was voltage-independent; normalized slope conductance for R117H measured at 50 mV positive and negative to the reversal potential was 0.74 ± 0.05 and 0.78 ± 0.05 ($n=8$), respectively relative to pH 7.3 (start). **METHODS.** Whole-cell currents were recorded from HeLa cells transiently expressing R117H as described^{3,27}. A List EPC-7 amplifier (Adams and List, Westbury, NY) was used for voltage-clamping and current amplification. A microcomputer (IBM AT compatible) and the pClamp software package (Axon Instruments), were used for data acquisition and analysis. The pipette (internal solution) contained (in mM): 120 *N*-methyl-D-glucamine (NMDG), 85 aspartic acid, 3 MgCl₂, 1 Cs-EGTA, 1 Mg-ATP, 5 TES (*N*-Tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid), pH 7.3 with HCl ([Cl⁻] 43 mM; [Ca²⁺]_{free} < 10^{-8} M). The bath solution contained (mM): 140 NaCl, 1.2 MgSO₄, 1.2 CaCl₂, 10 dextrose, 10 TES (pH 7.3 with NaOH) except that in **c** (external pH experiments) 5 mM CHES (2-[*N*-cyclohexylamino]ethanesulphonic acid) and 5 mM MES (2-(4-morpholino)-ethanesulphonic acid) replaced 10 mM TES in the bath solution and solutions were titrated to either pH 5.8, 7.3 or 8.8 with NaOH; these buffers had no significant effect on wild-type CFTR Cl⁻ currents (not shown). Experiments were conducted at 34–36°C. The established sign convention was used throughout. Liquid junction potentials and potentials at the tip of the patch-pipette were measured and *I-V* relationships corrected for the corresponding offset. Currents, filtered at 0.5 kHz and digitized at 2 kHz, have not been capacitance- or leakage-subtracted.

cAMP-dependent protein kinase (PKA) and, like wild-type CFTR, the mutants required cytosolic ATP. Thus, phosphorylation-dependent regulation through the regulatory (R) domain⁹ and ATP-dependent regulation through the nucleotide-binding domains¹⁰ appeared similar to that of wild-type CFTR. However, the biophysical properties of the mutants differed strikingly from those of wild type.

Figure 4a shows two important differences. First, single-channel current amplitude was reduced. The effect is not readily apparent by visual inspection for R117H, but for R334W and R347P, it was dramatic (Fig. 4a). To quantitate the reduction, we analysed single-channel $I-V$ relationships (Fig. 4b); we used a Cl^- concentration gradient (external $[\text{Cl}^-]$, 10 mM; internal $[\text{Cl}^-]$, 147 mM) to magnify the small current amplitude. Figure 4b (inset) shows that under these conditions, wild-type CFTR had a rectifying $I-V$ relationship with a reversal potential at about +60 mV, consistent with Cl^- selectivity. At negative voltages, where the $I-V$ relationship was linear, wild-type CFTR had a slope conductance of 7.86 ± 0.21 pS ($n=8$). The conductance for R117H was 6.76 ± 0.23 pS ($n=6$); although the decrease in conductance was small, it was statistically significant ($P=0.004$; Student's unpaired t -test). The conductance for R347P was more severely decreased at 2.34 ± 0.23 pS ($n=4$, $P<0.001$). We were never able to resolve discrete channel openings for R334W, even at -150 mV with a Cl^- concentration gradient, suggesting that its conductance was markedly reduced. We cannot, however, rule out the alternative explanation that the kinetics were markedly altered so that the time spent in the open state was decreased beyond resolution.

Second, the kinetics of R117H were altered: they were characterized by an apparent shortening of the time that channels were open (Fig. 4a). R347P appeared to have kinetics more like wild-type CFTR. We quantitated these changes by measuring the probability that the channel was in the open state (P_0). R117H had a P_0 less than one third that of wild-type CFTR (Fig. 4c). P_0 was voltage-independent, consistent with the time-independence of the whole-cell currents. We could not accurately measure the P_0 of R347P because of its very small single-channel conductance.

These data explain why R117H, R334W and R347P had a lower apical membrane Cl^- conductance (G_{apical}) than wild-type CFTR. G_{apical} is determined by the number of channels in the

apical membrane (N), the conductance of a single channel (g), and the probability that a single channel is open (P_0): $G_{\text{apical}} = NgP_0$. R347P appeared to open like wild type, but g was 30% of that of wild type; this explains a G_{apical} that was 33% of that of wild type. For R117H, P_0 was 28% and g was 86%; these values predict a G_{apical} of 24%, in reasonable agreement with the observed G_{apical} of 15%. We could not resolve single-channel currents for R334W, suggesting that g was very small and/or that P_0 was much reduced; in comparison, G_{apical} was only 4% of that of wild type. Because the decreased G_{apical} is explained by alterations in g and P_0 , the data suggest that N is the same for wild-type CFTR and these three mutants. The finding that these mutants are correctly processed (Fig. 1a) supports this conclusion and contrasts with the $\Delta F508$ mutant, which is not delivered to the apical membrane^{6,19,21}. Thus our results provide a molecular explanation for the quantitative decrease in cAMP-activated Cl^- current in the apical membrane.

Two mutations in M6 altered single-channel conductance: substitution of R334 by the bulky hydrophobic tryptophan severely reduced conductance, and substitution of R347 by proline, which can disrupt an α -helix, reduced conductance by two thirds. In addition, we had previously found that mutation of a basic amino acid in M6 to an acidic residue, K335E, altered anion selectivity³. The ability of multiple mutations within M6 to alter different specific properties of the conduction mechanism suggests that M6 contributes to the pore of the CFTR Cl^- channel. The finding that mutations in M1 (K95; ref. 3) and at the external surface of M2 (R117) also alter specific pore properties suggests that the permeation pathway may also involve this region of CFTR.

These observations have several implications for understanding and treating CF. First, they show that different classes of mutations disrupt CFTR Cl^- channel function in different ways and that the mechanism of dysfunction may determine the clinical phenotype. Some mutations, such as $\Delta F508$, disrupt normal processing and hence are missing from the apical membrane^{6,19-21}; such mutants generate no apical Cl^- current and are associated with severe disease. Other mutants are correctly processed; at least three of these (R117H, R334W and R347P) retain significant apical Cl^- channel function and are associated with a milder form of the disease. Thus the CF genotype determines the biochemical abnormality, which determines the

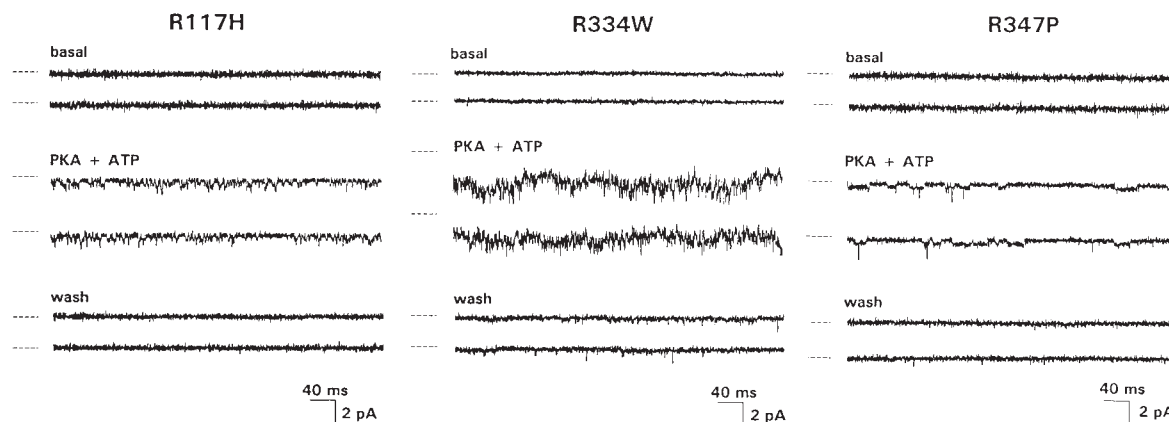


FIG. 3 Reversible activation of R117H, R334W and R347P by phosphorylation with cAMP-dependent protein kinase (PKA) (75 nM) and ATP (1 mM). No channel activity was observed in response to ATP before addition of PKA. Representative recordings are from excised inside-out membrane patches from HeLa cells transiently expressing either R117H, R334W or R347P; $n=8$ for each mutant. Voltage was -13 mV (R117H), -126 mV (R334W) and -130 mV (R347P), respectively. Dashed lines indicate the closed channel state and downward deflections correspond to channel openings. METHODS. Currents were recorded as described^{7,10,27}. The pipette (extracellular) solution contained (in mM): 140 NMDG, 140 aspartic acid, 5 CaCl_2 ,

2 MgSO_4 and 10 TES, pH 7.3 with Tris ($[\text{Cl}^-]$, 10 mM). The bath (intracellular) solution contained (mM): 140 NMDG, 3 MgCl_2 , 1 Cs-EGTA and 10 TES, pH 7.3 with HCl ($[\text{Cl}^-]$, 147 mM; $[\text{Ca}^{2+}]$ free $<10^{-8}$ M). Experiments were conducted at $34-36^\circ\text{C}$. Currents were filtered at 1 kHz and digitized at 10 kHz. Liquid junction potentials and potentials at the tip of patch-pipettes were measured and $I-V$ relationships corrected for the corresponding offset. No channel activity was observed in recordings from excised inside-out membrane patches from control virus-infected cells made under identical conditions ($n=4$).

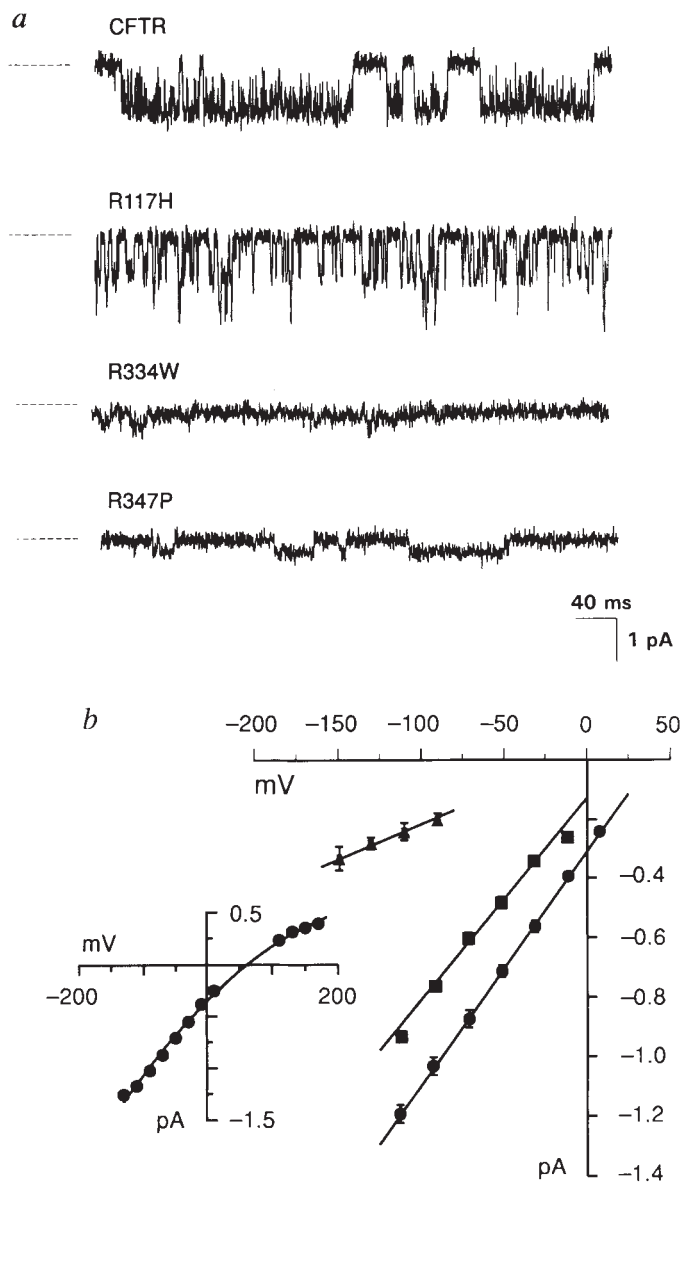


FIG. 4 R117H, R334W and R347P have altered single-channel properties. *a*, Representative single-channel recordings are from excised inside-out membrane patches from HeLa cells transiently expressing wild-type CFTR, R117H, R334W or R347P; currents were recorded as described in Fig. 3 legend. All recordings were obtained in presence of PKA (75 nM) and ATP (1 mM) in the intracellular solution. Voltage was -88 mV (CFTR), -93 mV (R117H), -97 mV (R334W) and -91 mV (R347P). The number of channels present in the R334W tracing could not be determined. *b*, Single-channel current-voltage relationships of CFTR (●), R117H (■) and R347P (▲). Data points are mean $\pm$ s.e.m. of 3–8 values at each voltage. Lines are mean slope conductance calculated from slope conductance from individual experiments. Inset shows I - V relationship for one experiment with wild-type CFTR to show Goldman-type rectification at positive voltages (expected $E_{Cl} = 71$ mV). *c*, Relationship between single-channel open-state probability (P_o) and voltage for CFTR (●) and R117H (■). Data are mean $\pm$ s.e.m.; $n = 3$ –4 for CFTR and $n = 2$ –3 for R117H.

METHODS. Single-channel current amplitudes were determined from the fit of gaussian distributions to current amplitude histograms; the fit of linear least-squares regression lines to single-channel current-voltage relationships was used to determine single-channel conductance. Although the conductance of R117H and R347P was decreased, even at filter frequencies of 2.5 kHz, we cannot exclude the possibility that the mutations induced a very rapid flicker that could not be resolved. P_o was measured in patches containing ≤ 5 channels in the presence of 75 nM PKA and 1 mM ATP. Number of channels in each patch was determined from the maximum number simultaneously open with 3 mM ATP in the intracellular solution. Data were analysed using the pClamp software package (Axon Instruments).

clinical phenotype. Second, the data explain the finding that 'mild' mutations are dominant in conferring disease phenotype¹⁷. For example, a compound heterozygote with R117H on one chromosome and $\Delta F508$ on the other would have significant residual Cl^- channel activity (our data would suggest 7.5% function per cell based on values of G_{apical} divided by two to account for expression from a single chromosome). Third, because R117H, R334W and R347P have normal regulation, interventions designed to increase the activity of mutant CFTR might have therapeutic efficacy in patients bearing such mutations. β -adrenergic agonists, which increase cellular levels of cAMP and can be delivered by inhalation, are candidates that may be worth investigation²⁶. □

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Myoblasts transferred to the limbs of embryos are committed to specific fibre fates

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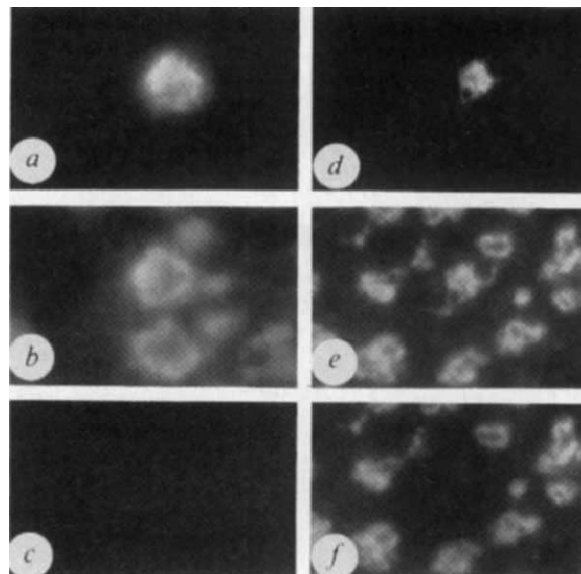
IN the limb bud of the 5-day-old avian embryo, when primary muscle fibre formation is beginning and before specific muscles appear, differences in the expression of fast and slow myosin heavy chain genes can be detected among primary fibres of the premuscle masses^{1,2}. Myoblasts that form colonies of fibres of specific types can be isolated from these limb buds³. To assess the role of myoblast commitment in specifying fibre types during embryonic development, we cloned myoblasts of specific types from embryonic and adult muscles, transfected them with a reporter gene, and transferred them into developing limb buds. After transfer, cloned myoblasts formed fibres in the limb with the same patterns of myosin heavy chain gene expression as the fibres they formed in cell culture. These results demonstrate that initial skeletal muscle fibre type diversity during avian limb development can originate, in part, from the commitment of distinct myoblast types to the formation of specific fibre types.

Adult avian skeletal muscle is composed of fast, slow, and fast/slow fibre types which express members of the fast, slow, or both fast and slow classes of myosin heavy chain (MHC) genes¹. This diversity in fibre types could initially arise from distinct myoblast cell lineages or from a single myoblast lineage which forms fibres that under the influence of extrinsic factors such as innervation or hormones express different MHCs. Fibre

type diversity in the limb is, in fact, evident before functional innervation^{1,4,5} of the dorsal and ventral premuscle masses of the developing limb, and it is possible that this initial fibre type diversity may originate in distinct myoblast populations present in embryonic limbs^{3,6-8}. Using monoclonal antibodies that specifically recognize fast and developmentally regulated slow MHC isoforms, limb buds were shown to contain embryonic myoblasts of two major types which, when cloned, formed fibres that expressed distinct subsets of MHC genes⁶⁻⁹.

To evaluate more critically the potential role of myoblast commitment to initial formation of skeletal muscle fibre types, myoblasts from embryonic day 4 or adult quail limbs were transfected with a reporter gene construct, cloned, passaged five times and injected into developing ED5 chick limb buds. The reporter gene construct, pNEOAH, carries the *Drosophila melanogaster* alcohol dehydrogenase (ADH) gene¹⁰ under constitutive transcriptional control of the Rous sarcoma virus long terminal repeat and simian virus 40 (SV40) polyadenylation sequences. pNEOAH also carries a neomycin resistance gene. Cells were transfected by calcium-phosphate precipitation, selected by G-418, and then cloned to obtain a homogeneous myoblast population. A portion of each cloned myoblast population was allowed to differentiate into fibres *in vitro*. These fibres were then immunostained with an anti-ADH polyclonal antibody, and monoclonal antibodies F59 and NA8, which specifically recognize fast MHCs^{1,6} and slow MHC (SMHC2 and 3) isoforms (E. Bandman, personal communication and western blotting, not shown; ref. 2), respectively. Myoblasts of specific clonal types were injected into embryonic day 5 (ED5) chick limb buds in which primary muscle fibres are just beginning to form in the premuscle masses of the proximal region of the bud. At ED10, when the host muscles were formed and innervated, and fibre types are well established, limbs were embedded in paraffin, sectioned, and immunostained with anti-ADH, F59 and NA8 antibodies. Fibres identified by the anti-ADH antibody were usually found in muscle-forming regions

FIG. 1 Sections of ED10 chick hind limbs containing fibres formed from embryonic (ED4) myoblast clone EF1 after transfer at ED5. Myoblast clone EF1 exclusively formed fibres *in vitro* that expressed only fast MHC isoforms. ADH-expressing fibres *in vivo* (a, d) reacted with anti-fast MHC monoclonal antibody F59 (b, e) but not anti-slow MHC (SMHC2 and 3) monoclonal antibody NA8 (c, f) whether the fibre formed in a fast (a, b, c) or fast and slow muscle (d, e, f). All sections were photographed at an original magnification of $\times 1,250$ using epifluorescence on a Zeiss Universal microscope. Variation in fibre diameter was dependent on individual muscles and location of the section along the muscle axis. Myoblast transfer into embryonic (ED5) chick limb buds was powered by a Picospritzer II (General Valve Corp.) injector set at 15–20 ms at 15–30 p.s.i. depending on the cell density. Typically, $1.0\text{--}2.0 \times 10^4$ cells were transferred into each limb bud. At ED10, limbs were embedded in paraffin, sectioned at a thickness of 10 μm , deparaffinized, rehydrated, and incubated in a blocking solution of 2% BSA, 5% horse serum in phosphate buffered saline (PBS) for 2 h. Sections were then incubated in the following antibodies for 1 h each with three washes of PBS between each incubation: Anti-slow MHC monoclonal antibody NA8 (kindly provided by E. Bandman; it has been shown that NA8 reacts with the same quail muscle fibres and same MHC bands on immunoblots as antibody S58 (refs 1, 2, 9)) and anti-ADH antibody diluted 1:1000 and 1:2000, respectively, in blocking solution; Texas red-conjugated anti-mouse IgG and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (Vector Labs) diluted 1:100 in blocking solution; biotinylated anti-fast MHC monoclonal antibody F59 (refs 1, 6) diluted 1:100 in blocking solution; AMCA (7-amino-4-methylcoumarin-3-acetic acid)-conjugated avidin (Vector Labs) diluted 1:100 in blocking solution. Sections were washed with PBS, then 2.5% diazabicyclooctane (DABCO) in 90% glycerol and coverslips were applied. Anti-ADH polyclonal antibody was generated by immunization of rabbits with alcohol dehydrogenase purified from *Drosophila melanogaster*¹⁸. Partially purified ADH was electrophoresed in a 10% SDS-PAGE. The ADH band was cut from the preparative gel and eluted overnight at 100 V in a Tris-glycine gel running buffer (no SDS) using an Elutrap ElectroEluter (Schleicher and Schuell). Rabbits were immunized with 350 μg purified ADH mixed with complete Freund's adjuvant. After 3 weeks, rabbits were boosted with 150 μg purified



ADH mixed with incomplete Freund's adjuvant. Rabbits were bled 1 week later and serum was titred against preimmune serum on dot blots of purified ADH. ADH polyclonal antibody was affinity purified on an ADH-Affi-Gel 10 (Bio-Rad) column according to manufacturer's instructions. Purified IgG was obtained from F59 monoclonal supernatant by an ABx (JT Baker) column and precipitated by ammonium sulphate. Purified IgG was biotinylated using *N*-hydrozysuccinimide biotin (Vector Labs) according to manufacturer's instructions.

TABLE 1 Myosin heavy chain analysis of ADH-expressing fibres

Myoblast type	Limbs analysed*	ADH expressing fibres in analysed limbs	ADH-expressing fibres in sectioned limbs	ADH- and fast MHC-expressing fibres	ADH- and slow MHC-expressing fibres†	Per cent fibres of one type
Embryonic fast, EF1	11	17	8	8	0	100
Embryonic fast/slow, ESF1	29	37	13	13	13	100
Adult fast/slow, ASF1, ASF2	22	46	25	25	25	100

* Limbs were analysed for ADH-expressing fibres in whole-mount preparations and in sections 1–5 days after cell transfer (ED6–10).

† Fibres expressed slow MHC2 and/or MHC3 ref. 2.

but occasionally were located in nonmuscle-forming regions of the limb. Longitudinal sections of limbs (not shown) verified that transferred myoblasts formed morphologically identifiable muscle fibres.

Embryonic (ED4) quail myoblast clones EF1 or ESF1, characterized by the patterns of MHC genes expressed in fibres they formed in cell culture, were transferred into ED5 chick limb buds. Myoblast clone EF1 formed fibres that expressed only fast MHC(s) (F59 reactive) but no slow MHCs (NA8 unreactive) *in vitro*. Limbs into which myoblast clone EF1 was transferred always contained fibres that expressed ADH, fast MHC(s), but no slow MHCs (Fig. 1). Although ADH-expressing fibres were located in host muscles containing either fast or fast and slow fibres, the pattern of MHC gene expression within fibres formed by the transferred myoblasts remained unaltered. Myoblast clone ESF1, which formed fibres that expressed both fast MHC(s) and slow MHCs *in vitro*, formed fibres in the host muscles which, when assessed at ED10, always expressed both fast MHC(s) and slow MHCs (Fig. 2). This pattern of MHC gene expression in fibres formed by myoblast clone ESF1 remained the same whether or not the fibre was located in host muscles expressing slow MHCs. Transferred cloned embryonic myoblasts, therefore formed muscle fibres that expressed MHCs with similar reactivities to anti-fast (F59) and anti-slow (NA8) monoclonal antibodies whether they formed *in vivo* or *in vitro*.

Adult myoblasts (satellite cells) from adult avian skeletal muscle have been shown to be of different types expressing

discrete subsets of MHC genes when they differentiate into fibres in clonal cell culture¹¹. Adult satellite cells were isolated, transfected with pNEOADH, and cloned. The satellite cell clones ASF1 and ASF2 formed fibres *in vitro* that expressed both fast MHC(s) and slow MHC(s). Each clone was transferred into ED5 chick limb buds which were allowed to develop until ED10. Limbs were sectioned and immunostained with anti-ADH, and F59 and NA8 monoclonal antibodies (Fig. 3). All fibres formed by transferred adult myoblasts expressed both fast MHC(s) and slow MHC(s). This pattern of expression was observed in ADH-expressing fibres whether these fibres were located in muscles containing fast and slow or only fast MHC-expressing fibres.

In all the experiments, each fibre formed from transferred myoblasts *in vivo* expressed the same MHC genes as fibres formed by the same myoblast clone *in vitro* (Table 1). The patterns of MHC gene expression in fibres formed from transferred myoblasts were those expressed *in vitro* despite influences present in the embryo and embryonic muscle-forming regions. Therefore, it is likely that the intrinsic myogenic programmes that establish myotube diversity *in vitro* are also initially operative *in vivo* in newly forming fibres during the early embryonic development of the limb. That fusion of quail myoblasts (ESF1) with normal chick fetal myoblasts does not necessarily alter either ADH or slow MHC expression was investigated by mixing these two types of myoblasts in cell culture. Chimaeric myotubes formed containing chick and quail nuclei and the fibres

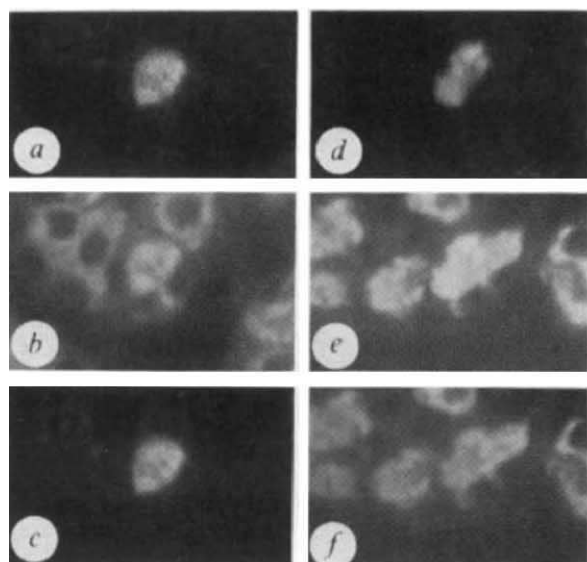


FIG. 2 Sections of ED10 chick hind limbs containing fibres formed from embryonic (ED4) myoblast clone ESF1 after transfer at ED5. Myoblast clone ESF1 exclusively formed fibres *in vitro* expressing both fast and slow MHC isoforms. ADH expressing fibres *in vivo* (a, d) reacted with anti-fast MHC monoclonal antibody F59 (b, e) and anti-slow MHC monoclonal antibody NA8 (c, f) whether the fibre formed in host muscle regions with fibres synthesizing only fast MHC(s) (a–c) or fast and slow MHCs (d–f).

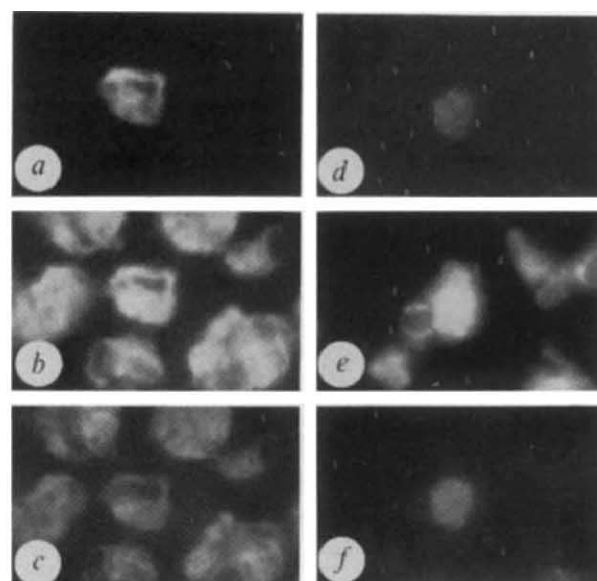


FIG. 3 Sections of ED10 chick limbs containing fibres formed from adult myoblast clones ASF1 (a–c) and ASF2 (d–f) after transfer at ED5. These myoblast clones exclusively formed fibres *in vitro* that expressed both fast and slow MHC isoforms. ADH expressing fibres *in vivo* (a, d) synthesized fast (b, e) and slow (c, f) MHCs whether these fibres formed in host muscle regions containing fibres expressing fast and slow MHCs (a–c) or only fast MHC(s) (d–f).

expressed both ADH and the 238 MHC characteristic of the fibres formed from ESF1 myoblasts alone.

It is clear that factors extrinsic to muscle fibres such as innervation^{1,12-14}, activity^{1,14} and hormones^{15,16} can influence muscle fibre development and MHC gene expression beyond the embryonic period of development. These extrinsic factors, however, have not been shown to be operative at stages of early limb development when diversity of skeletal muscle fibres first appears. Therefore, there are either other factors extrinsic to muscle fibres that dictate the diverse patterns of MHC gene expression in fibres during early myogenesis, or, more likely, myogenic programmes intrinsic to distinct myoblast types acting with local factors govern the initial differentiation of muscle fibres. Here we have shown that individual clonal myoblast populations follow a similar myogenic pathway both *in vivo*

and *in vitro* despite different muscle fibre environments *in vivo*.

It has been reported¹⁷ that transfer of clonal myoblasts into limb muscles in the postnatal period where patterns of MHC gene expression within fibres and functional innervation of fibres are well established, did not alter the phenotype of fibres into which transferred myoblasts had fused. This result is not in conflict with the present report because the former study examined the ability of different types of myoblasts to fuse into existing multinucleated fibres and the ability of the fusing myoblast nuclei to be entrained by already established MHC gene regulatory mechanisms within established fibre types. The results reported here demonstrate that intrinsic myogenic programmes within each type of myoblast can provide a mechanism for formation of diverse muscle fibre types during early avian limb development. □

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Principles of locomotion for simple-shaped cells

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MOVING cells display a variety of shapes and modes of locomotion¹, but it is not clear how motility at the molecular level relates to the locomotion of a whole cell, a problem compounded in studies of cells with complex shapes²⁻⁵. A striking feature of fish epidermal keratocyte locomotion is its apparent simplicity⁶. Here we present a kinematic description of locomotion which is consistent with the semicircular shape and persistent 'gliding' motion of fish epidermal keratocytes. We propose that extension of the front and retraction of the rear of these cells occurs perpendicularly to the cell edge, and that a graded distribution of extension and retraction rates along the cell margin maintains cell shape and size during locomotion. Evidence for this description is provided by the predicted circumferential motion of lamellar features and the curvature of 'photo-marked' lines within specific molecular components of moving keratocytes. Our description relates the dynamics of molecular assemblies to the movement of a whole cell.

The close association between the semicircular shape of keratocytes and their 'gliding' mode of locomotion suggests that cell shape arises from the same process that drives locomotion. The following kinematic description, termed the graded radial extension (GRE) model, defines the magnitude and direction of lamellar extension or retraction required to maintain the semicircular shape and constant velocity of a moving cell (Fig. 1a). We propose that extension of the leading edge, represented by the semicircle EAQ, occurs along radii perpendicular to the cell edge at every point along EAQ. In addition the distance extended per unit time interval, or the extension rate, decreases

with increasing angular displacement from the maximum that occurs at the cell apex, A (Fig. 1a). Extension is counterbalanced by a graded retraction perpendicular to the rear cell margin, represented by EBKQ (Fig. 1a). Points E and Q, termed equilibrium points, are regions where there is no net extension or retraction. A line drawn between these points represents an imaginary equator which divides net extending regions of the lamella from net retracting regions. This graded, symmetrical arrangement of extension and retraction rates perpendicular to the cell edge results in forward translocation of the cell together with a maintenance of its size and shape. A simpler alternative to the GRE model is the parallel extension model (Fig. 1b), in which all regions along the cell margin advance with the same speed and direction. Note that the process of cell spreading is a form of radial extension distinct from the GRE model. Spreading is defined here as the equal extension of the cell edge in all directions which does not result in cell movement.

The GRE model predicts that regions on extending or retracting edges will move along the circumference of the cell during locomotion (Fig. 1c). For example, region P on the right side of the extending edge AQ moves along the circumference in a clockwise direction. Similarly, regions on the left side of the extending edge AE move along the circumference in an anti-clockwise direction. This movement is termed circumferential motion (CM). It is the resultant motion arising from either graded radial extension or retraction and it can occur within any area of the hypothetical keratocyte lamella. CM is not predicted by the parallel extension model or by the process of spreading. Thus the detection of CM in moving keratocytes is a specific test for the operation of the GRE model.

Most rapidly moving, semicircular-shaped keratocytes observed by phase-contrast microscopy have several phase-dense ridges arranged radially within the lamella (Fig. 2). These ridges represent cytoplasmic thickenings within the lamella, as shown by scanning electron microscopy, and, in live cells, by the increased fluorescence of a nonspecific cytoplasmic stain in these regions (unpublished observations). Lamellar ridges are transient features that exist for about 10-60 s before disappearing within retracting regions of the lamella (Fig. 2a-c). As the cell advances, the distal tips of most lamellar ridges elongate perpendicular to and at the same rate as the extending edge. In

addition, these regions often become broader and flatter in regions where lamellar extension is more rapid (Fig. 2*b*). As the lamellar ridges elongate, they rotate along the circumference of the cell (Fig. 2*a-c*). Ridges to the right of the cell apex rotate in a clockwise direction while those on the left rotate in an anticlockwise direction, in accordance with the GRE model. If the position of a ridge is plotted with respect to the substratum, a curved trajectory is produced (Fig. 2*d*) which is consistent with ridge elongation occurring in a graded radial manner (Fig. 1*c*). The CM of lamellar ridges with respect to the cell surface (Fig. 2*e*) is markedly similar to the behaviour of simulated lamellar ridges (Fig. 1*d*). A quantitative analysis of ridge motion in six different cells shows that virtually all ridges behave as predicted by the GRE model (Fig. 2*f*).

Evidence for graded radial retraction is provided by the formation of curved retraction fibres at the rear edge of a moving keratocyte (Fig. 2*g*). Retraction fibres form from small regions of lamella that remain attached to the substratum. As the cell advances, these regions become elongated while maintaining their attachment to the substratum. Thus the elongating retraction fibres trace the course of a small, retracting region of the cell margin. A striking similarity can be seen between the curvature and length of these fibres compared with simulated fibres produced by graded radial retraction (Fig. 2*g, h*).

The effect of a graded radial extension on cell surface com-

ponents is shown by the progressive curvature of a line photobleached across an extending keratocyte lamella, stained with a rhodamine-labelled, monovalent lectin (Fig. 3*a-c*). In addition, the line is generally displaced with respect to the substratum in the direction of cell movement. The photobleached line becomes broader and less distinct owing to lateral diffusion of the lectin-receptor complexes (Fig. 3*d-f*). But the curvature of the photobleached line matches that of a line simulated according to the rules of graded radial extension (Fig. 3*g-i*). In both cases a velocity component perpendicular to the direction of cell movement causes the line to curve. This curvature will be similar to that of the cell margin because the graded distribution of lamellar extension rates maintains a semicircular cell shape. In contrast, no curvature of the photobleached line would be expected according to the parallel extension model.

In another series of experiments, caged fluorescent actin was photoactivated in a line across the extending lamella of a moving keratocyte (Fig. 4*a-c*). At increasing intervals after photoactivation the line becomes progressively curved in retracting regions of the lamella only (Fig. 4*b, c*). This behaviour is also predicted by simulation of photoactivated lines according to the rules of graded radial retraction (Fig. 4*d-f*). Both observed and simulated lines remain stationary with respect to the substratum within net extending regions of the lamella⁷. But as the cell advances, the line penetrates further into retracting regions of

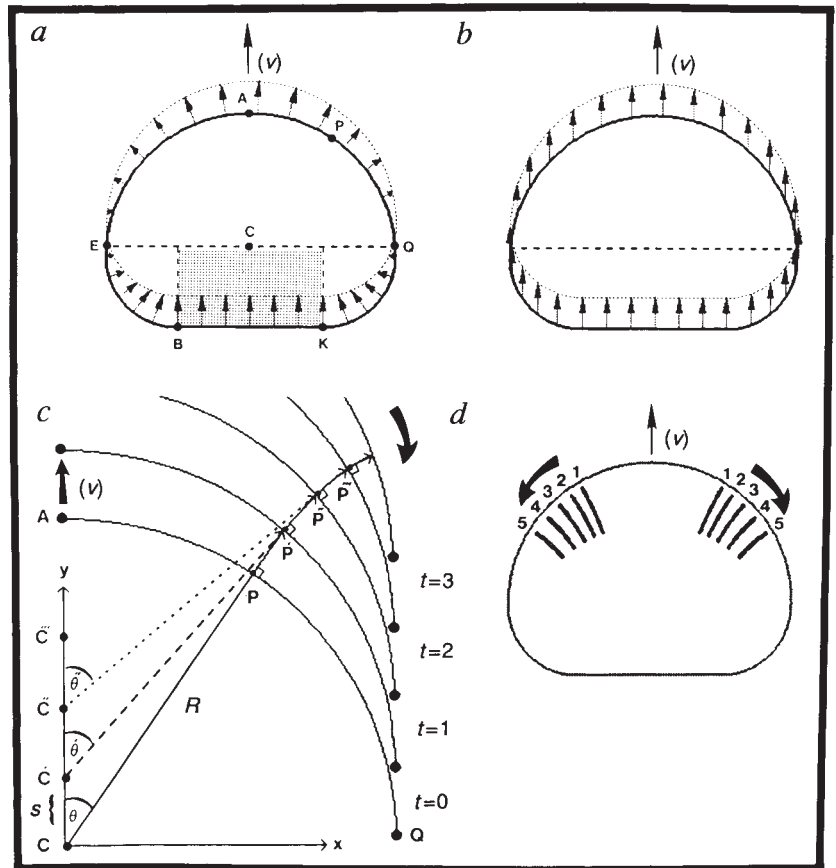
FIG. 1 *a*, An idealized shape of a motile keratocyte moving according to the GRE model. Its size represents a keratocyte that is about 25 μm long and 33 μm wide with a centre at C. Lamellar extension and retraction occur perpendicularly to the cell edge (arrows). In addition, these are graded along the cell margin so that cell size and shape is maintained at its subsequent position (dotted line). The same position dependence of extension and retraction rates holds for regions within the plane of the lamella. Thus the velocity of cell movement (V) is the net result of a specific organization of extension and retraction rates within the lamella. The rectangular area (stippled) represents the nuclear region of the cell which is assumed to advance with the rest of the cell. *b*, An idealized keratocyte moving according to the parallel extension model. The speed and direction (arrows) of lamellar extension (or retraction) occurs parallel to the velocity (V) of the whole cell. In contrast to *a*, cell movement results from all regions of the cell having the same velocity. *c*, Simulation of graded radial extension. Consider an arbitrary point P on the right side of the extending cell margin of an idealized keratocyte (see *a*) advancing at constant velocity V . At $t=0$, P lies on a semicircle of radius R (solid line) and origin C located at the centre of a cartesian coordinate system fixed with respect to the substratum. Points on this semicircle are given by $x^2 + y^2 = R^2$, where $0 \leq x \leq R$ and $0 \leq y \leq R$. During the interval $0 \leq t \leq 1$, the semicircle has moved a small distance s (where $0 < s < R/10$) along the y axis such that its origin C has moved to C'. The radius R remains constant (dashed line). At $t=1$, points on this semicircle are given by

$$x'^2 + (y' - s)^2 = R^2 \quad (1)$$

In addition, P extends perpendicularly to the cell edge to its new position P' along a straight line CP given by

$$y = mx \quad (2)$$

where $m = \tan(90^\circ - \theta)$ and θ is the angle between CP and the y axis. The coordinates (x' , y') of P' are given by simultaneous solution of equations (1) and (2) to yield $x' = [ms + (R^2(1+m^2) - s^2)^{1/2}]/(1+m^2)$, and $y' = [m^2s + m(R^2(1+m^2) - s^2)^{1/2}]/(1+m^2)$. The new angle that C'P' makes with the y axis is $\theta' = 90^\circ - \arctan((y' - s)/x')$. By repeating this calculation, the successive positions of P, P' and P'' may be found for $t=2$ and $t=3$, with respect to either the substratum or cell. Note that the successive positions of P trace a curved trajectory with respect to the substratum in addition to moving along



the circumference of the cell (curved arrow). The change in position of any point within the lamella may be found by applying the same rules. *d*, The CM (curved arrows) of two simulated lamellar ridges on either side of the extending lamella are shown with respect to the cell frame. The size and velocity of the cell are similar to that of the cell shown in Fig. 2*a*. Successive ridge positions (1-5) are shown after every 12 s of locomotion.

the lamella. Thus line curvature begins at the point where it enters the retracting region of the lamella. The progressive curvature of both observed and simulated lines is in accord with an increasing rate of graded radial retraction at increasing angular displacements away from the equator (Fig. 1a). Line curvature is not predicted by retraction of the lamella parallel to the direction of cell movement.

Evidence for graded radial extension and retraction can both be explained in terms of actin filament dynamics. Extension of

the keratocyte lamella is directly associated with the polymerization of actin predominately at the leading edge⁷, as shown by the behaviour of a photoactivated actin line in this region (Fig. 4a-c). Evidence for actin polymerization at the edge of the extending lamellae exists in other cell types⁸⁻¹⁰. Furthermore, in motile mouse macrophages, the rate of lamellar extension perpendicular to the cell edge is directly proportional to the width of the actin filament meshwork within the lamella¹¹. Therefore, the graded radial extension of the keratocyte lamella

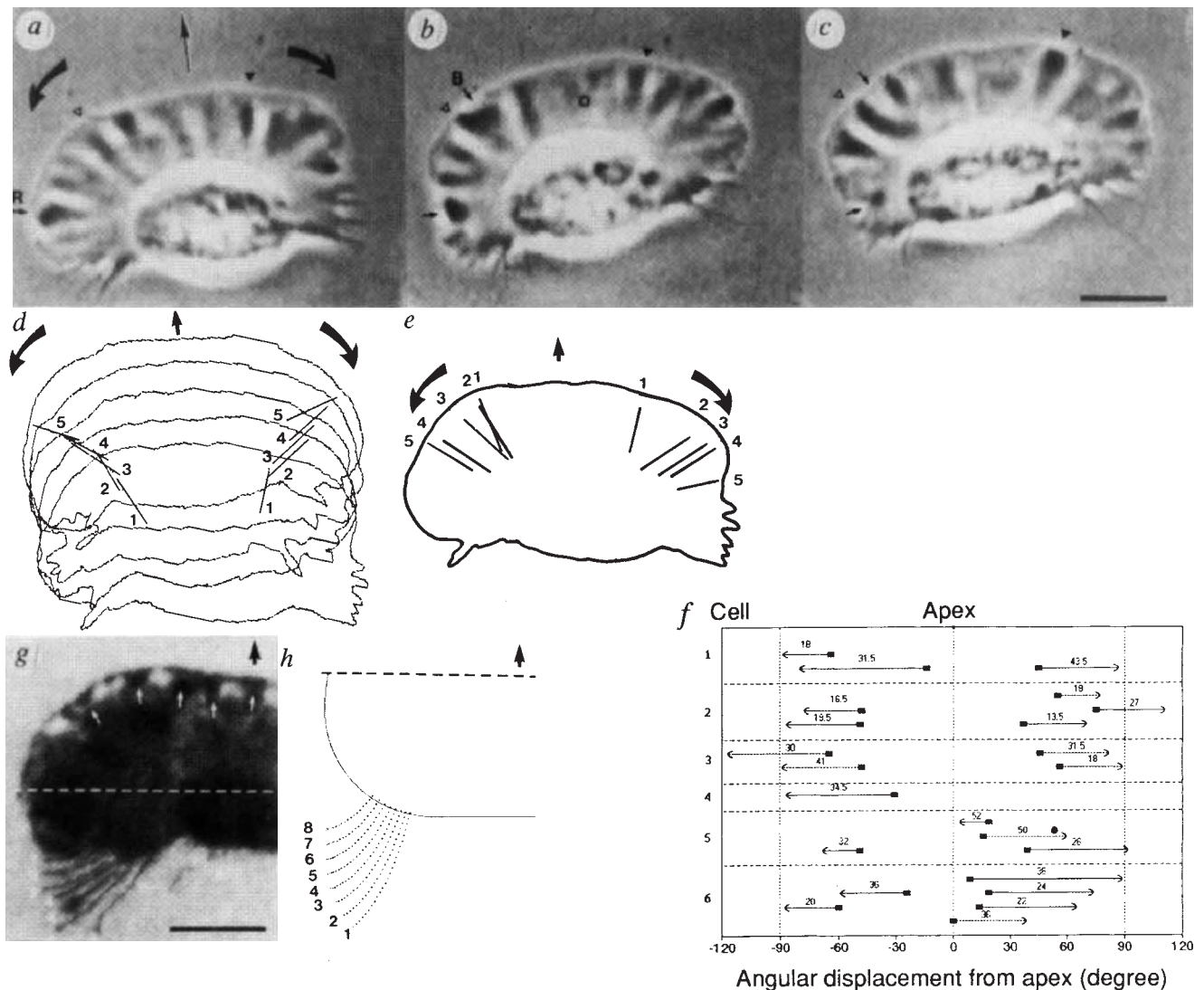


FIG. 2 a-c, A series of video-enhanced phase-contrast images, taken at 12-s intervals, of a single keratocyte moving at $16.6 \mu\text{m min}^{-1}$ in the direction of the straight arrow. CM of lamellar ridges (curved arrows) is seen to the left (triangle) and right (filled triangle) of the extending lamella. Lamellar ridges can form from the bifurcation of a broadened, pre-existing ridge (B) or arise spontaneously (circle). Ridges shorten and disappear within retracting regions of the lamella (R). Keratocytes were isolated from scales of goldfish, *Carassius auratus*²⁰ and cultured in RPMI medium with 10% fetal calf serum in a 'cover glass sandwich'²¹. A low-light-level ISIT camera (Dage MTI) and the Image I imaging system (Universal Imaging) were used to acquire, process and store images on a Panasonic optical memory disc recorder. Sequences of about 100 images were generally recorded at intervals of 1-2 frames per second. Scale bar, $10 \mu\text{m}$. d, The changes in position of the lamellar ridges in a (straight lines) are shown with respect to the substratum, together with a cell outline, every 12 s. e, The CM (curved arrows) of the lamellar ridges in d are shown with respect to the cell. Sequential ridge positions (1-5) are obtained by subtracting the cell's forward component of motion from each ridge position in d. Note the similarity in behaviour of these ridges to that predicted for simulated ridges according to the GRE model in Fig. 1d. f, The CM of 12 lamellar ridges with respect

to the cell frame shows that in virtually all cases they behave as predicted by the GRE model (Fig. 1d). Thus ridges on the right of an extending lamella rotate in a clockwise direction while those on the left rotate anticlockwise. Arrows indicate the direction and range of rotation (filled square indicates initial position); numbers indicate the duration of rotation in seconds. Image 1 software was used to record the angular position of one or more ridges with respect to an x axis fixed with respect to the substratum. The change in angular position of a ridge with respect to the cell surface was obtained and normalized for both cell speed and direction of locomotion. g, Video-enhanced interference reflection image of a moving keratocyte (bold arrow) showing the formation of curved retraction fibres (numbered in order of decreasing fibre length). The curvature and orientation of fibres (such as 2 and 5) are consistent with their formation being due to a graded radial retraction. The periodic arrangement of close cell-substratum contact regions (arrows) along the extending edge appears to cause a pleating of the lamella to form the ridges seen by phase-contrast microscopy (unpublished observation). h, Simulated retraction fibres are produced by applying the rules of graded radial retraction to eight small regions of the retracting cell margin. Each fibre (numbered as in g) is composed of a series of dots that trace the path of a single retracting region with respect to the substratum.

may be associated with a graded radial rate of actin polymerization along the leading edge.

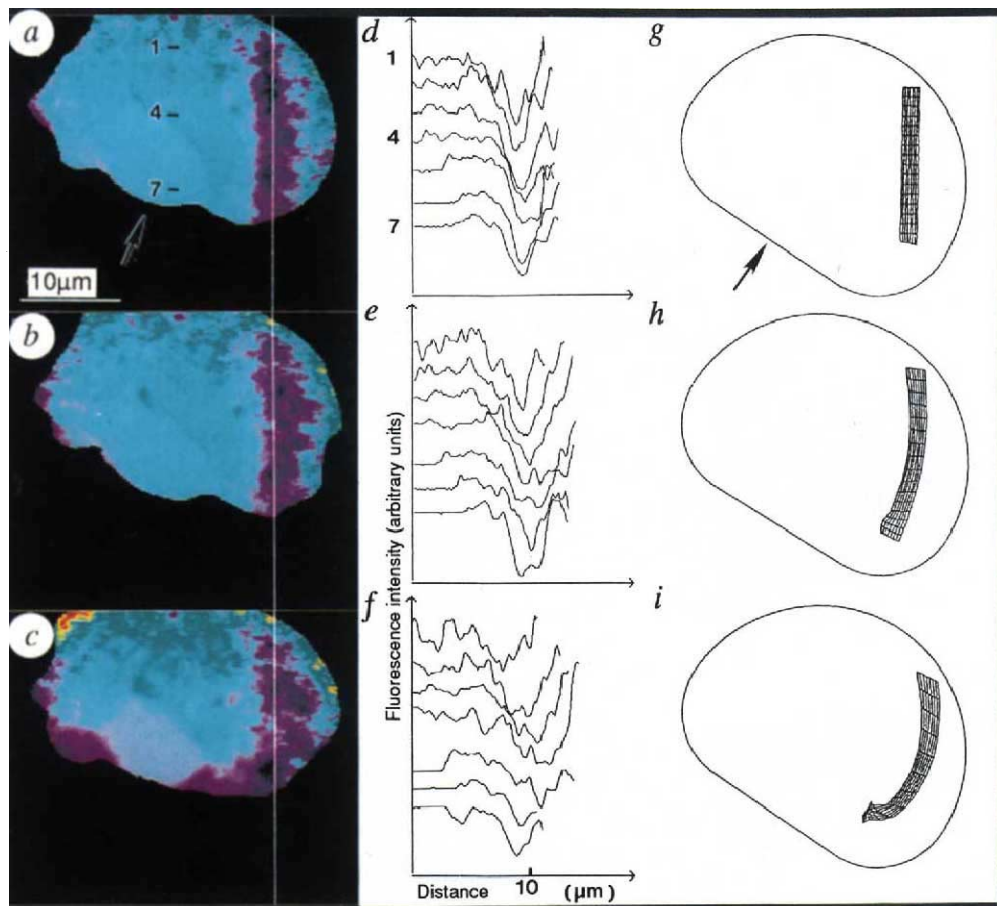
As actin polymerization at the leading edge plays a major role in cell movement, it is also likely to be involved in driving the bulk movements of the plasma membrane. It has been shown previously in keratocytes that lectin-receptor complexes advance passively with the moving cell¹² in the same way as was found for the plasma membrane in motile polymorphonuclear leukocytes¹³. Thus the curvature of photobleached lines across the lamella of lectin-stained keratocytes may be regarded as the indirect effect of a graded radial rate of actin polymerization at the extending edge. Indeed, in moving keratocytes, concanavalin A-coated particles display CM at the edge of the extending lamella (D. F. Kucik, personal communication). Similarly, the CM of lamellar ridges may also be regarded as an indirect consequence of a graded radial rate of actin polymerization at the leading edge.

The curvature of photoactivated actin lines indicates that a graded radial retraction of actin filaments is not limited to the rear edge of the keratocyte but occurs throughout the rear of the lamella. But the rate of actin depolymerization is constant within different regions of the keratocyte lamella⁷, as is the

distribution of myosin II staining (unpublished observation). These findings suggest a uniform contractility of the actin filament meshwork within all regions of the keratocyte lamella. Under such circumstances, the rear edge of the keratocyte lamella would retract at the same rate in all directions unless opposed by a graded radial extension. Thus differences in the regulation of actin filament dynamics may account for its role in both the extension and retraction of the keratocyte lamella. This is consistent with the suggested dual role of actin filament dynamics in the locomotion of other cell types^{3,14,15}.

The principles of locomotion described by the GRE model are evident in a variety of cell types. In simple-shaped cells, behaviour analogous to CM is shown by cytoplasmic swellings moving along the extending edge of amphibian keratocytes¹⁶. The locomotion of semicircular-shaped nematode sperm is associated with a graded elongation at the tips of non-actin-containing microfilaments oriented perpendicular to the cell edge¹⁷. The movement of more complex-shaped cells such as chicken growth cones is accompanied by the formation of microspikes which move along the cell circumference¹⁸. Furthermore, the formation of curved retraction fibres in macrophages is indicative of a graded radial retraction¹⁹. Thus the GRE model

FIG. 3 *a-c*, A sequence of ratio images at 200 ms, 2 s and 6 s after photobleaching. Progressive curvature can be seen of a line photobleached across the extending lamella of a keratocyte moving with a velocity of $30 \mu\text{m min}^{-1}$ (arrow) that is stained with a rhodamine-labelled lectin. The vertical white line that passes through the middle of the photobleached line in *a* represents a stationary marker with respect to the substratum. The slight line curvature in *b* is more pronounced in *c*. *d-f*, A series of 7 stacked fluorescence intensity profiles obtained from horizontal lines across the cell in the positions corresponding to those indicated by numbers 1, 4 and 7 in *a*. *d*, The region of the photobleached line is shown by a steep-sided trough in the fluorescence intensity profiles. Regions of lowest intensity lie along a vertical line corresponding to the middle of the bleached line in *a*. Progressive curvature of the photobleached line is shown by the relative change in position of the troughs in *e* and *f*, such that the regions of lowest fluorescence intensity lie along a curve. The sides of the troughs in *e* and *f* become shallower owing to diffusional recovery without affecting the position of the region of lowest intensity. *g-i*, Simulated behaviour of a photobleached line according to the GRE model reproduces the behaviour of the observed photobleached line. Photobleaching experiments were performed as described¹³ at room temperature. Keratocytes were stained with $20 \mu\text{g ml}^{-1}$ rhodamine-labelled, monovalent snail lectin (*Helix aspersa*) in PBS for 7 min, which resulted in a uniform staining of the cell surface with no impairment of cell motility. No patching or capping of lectin was observed, as shown by the maintenance of uniform lamellar staining during locomotion. Each post-bleach image was aligned with the pre-bleach image before producing a ratio image to remove any artefacts due to cell motion. Ratio imaging allows better visualization of the bleached line, as well as serving as a shading correction for the original fluorescence image. Ratio images are pseudocoloured to enhance subtle variations in fluorescence intensity. Cell outlines were



superimposed on ratio images and the background set to black. The behaviour of the photobleached line was simulated by applying the GRE model to points on the vertices of a bar-shaped grid across the extending lamella of a simulated cell moving at the same velocity (arrow) as in *a*. The simulated line is equivalent in size and orientation to the bleached line in *a*. Note that simulations of the GRE model do not account for redistribution of material within the lamella once it reaches regions corresponding to vertical lines at B and K (Fig. 1*a*). These regions represent 'singularities' where material will accumulate if simulations are continued to their end point.

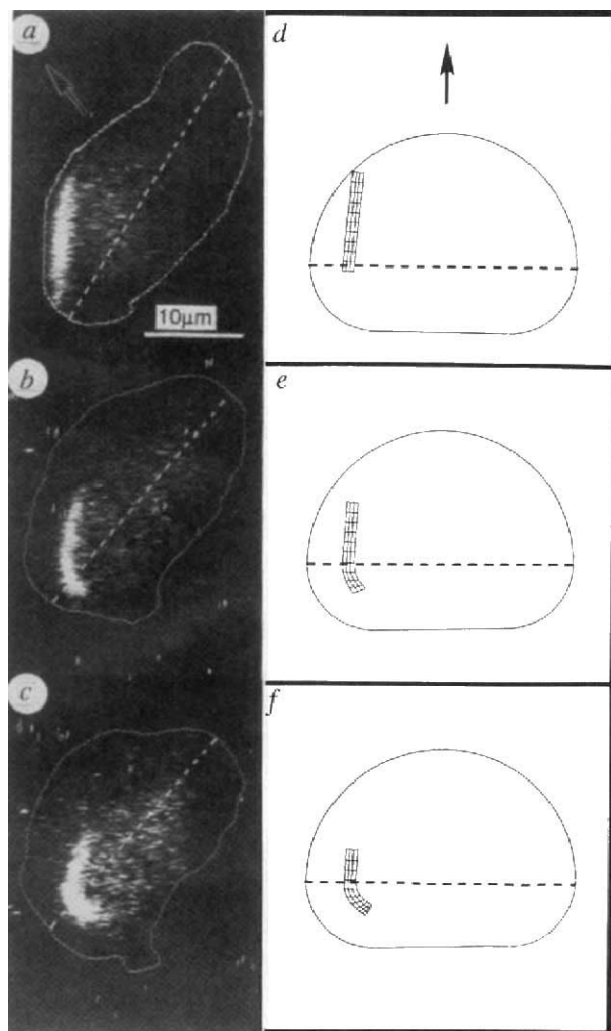


FIG. 4 *a-c*, A sequence of fluorescence images at ~0, 2 and 3 min, respectively, after photoactivation of a fluorescent line across the extending lamella of a keratocyte, cooled to 15 °C, moving at speed $3.40 \mu\text{m min}^{-1}$ (arrow). A cell outline from a corresponding phase image is superimposed on each image. The photoactivated line becomes progressively curved in retracting regions only. Note the similarity between this behaviour and that of a simulated actin bar. The kinetics of line curvature are dependent on cell speed. As a result of cooling, cells in this experiment are moving 10 times slower than those in Fig. 3. Therefore curvature of photoactivated lines occurs more slowly than photobleached lines. *d-f*, A simulated photoactivated actin bar becomes progressively curved, starting at the point where it crosses the equator (dotted line). Photoactivation experiments were performed as described⁷. Simulation of the photoactivated actin bar is made by assuming that it is stationary with respect to the substratum⁷, except in net retracting regions. The rules for graded radial retraction are then applied to points at the vertices of a bar-shaped grid within retracting regions of the lamella. Cell size and speed are similar to that of the cell in *a*.

describes kinematic principles for the locomotion of simple-shaped cells. It establishes a basis for understanding how molecular dynamics is related to the movement of a whole cell. □

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The protein Sex-lethal antagonizes the splicing factor U2AF to regulate alternative splicing of *transformer* pre-mRNA

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SOMATIC sexual differentiation in *Drosophila melanogaster* involves a cascade of regulated splicing events^{1,2} and provides an attractive model system for the analysis of alternative splicing mechanisms. The protein Sex-lethal (Sxl)^{3,4} activates a female-specific 3' splice site in the first intron of *transformer* (*tra*) pre-mRNA while repressing an alternative non-sex-specific site⁵⁻⁷. We have developed an *in vitro* system that recapitulates this regulation in a manner consistent with genetic, transfection and fly transformation studies⁴⁻⁸. Using this system, we have determined the molecular basis of the splice site switch. Here we show that Sxl inhibits splicing to the non-sex-specific (default) site by specifically binding to its polypyrimidine tract, blocking the binding of the essential splicing factor U2AF. This enables U2AF to activate the lower-affinity female-specific site. A splicing 'effector' domain present in U2AF but absent from Sxl accounts for the different activities of these two polypyrimidine-tract-binding proteins: addition of the U2AF effector domain to Sxl converts it from a splicing repressor to an activator and renders it unable to mediate splice-site switching.

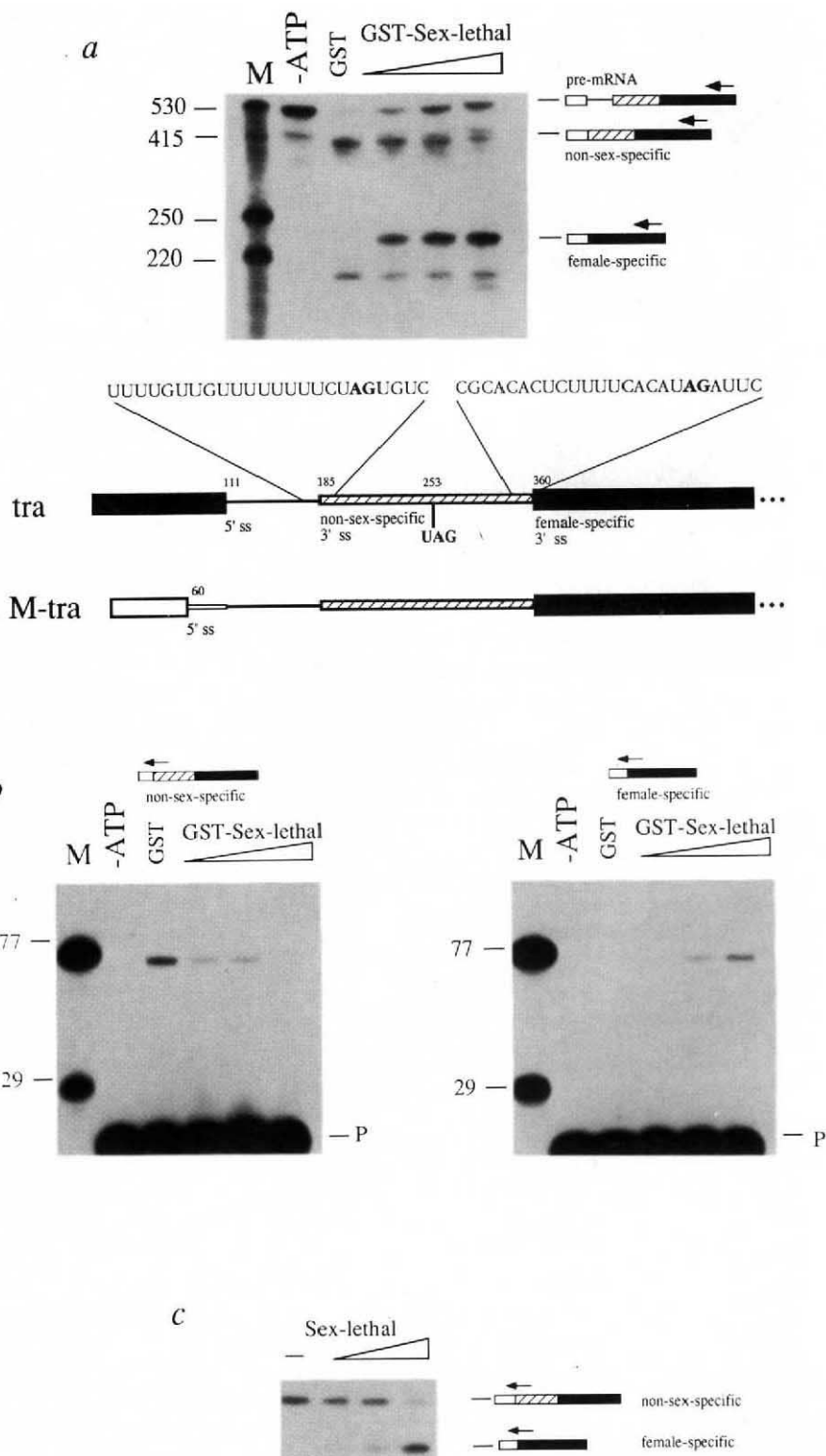
Experiments in transgenic flies have shown that only the region encompassing the two alternative 3' splice sites of *tra* is required for Sxl regulation⁷. To overcome the low *in vitro* splicing efficiency of the *tra* pre-mRNA (data not shown), we constructed a derivative (M-*tra*) containing the two alternative 3' splice sites of *tra* joined to the 5' splice site of the adenovirus major late gene (Ad ML) (Fig. 1a bottom). Splicing was analysed by primer-extension using primers complementary to sequences in the second exon (Fig. 1a) or those spanning the junctions of the two spliced mRNAs (Fig. 1b). Under standard conditions (with ATP), splicing was exclusively to the non-sex-specific 3' splice site. But upon addition of a glutathione-S-transferase (GST)-Sxl fusion protein, there was also splicing to the female-specific site. As the concentration of GST-Sxl was raised, splicing to the female-specific site increased and splicing to the non-sex-specific site decreased coordinately. Identical results were obtained with a Sxl protein derived from protease cleavage of GST-Sxl (Fig. 1c), or when *Drosophila* rather than HeLa cell nuclear extracts were used (data not shown). We conclude that our *in vitro* system reproduces the Sxl-dependent regulation of *tra* alternative 3' splice site choice observed *in vivo*⁴⁻⁸.

Studies in both transgenic flies and *Drosophila* cells in tissue culture have suggested that the site of Sxl action is the polypyrimidine tract of the non-sex-specific 3' splice site region^{7,8}. The pyrimidine tract is the binding site for the essential splicing factor U2 small nuclear ribonucleoprotein particle (snRNP) auxiliary factor (U2AF)⁹. U2AF is required for the ATP-dependent binding of U2 snRNP to the pre-mRNA branch point¹⁰,

an early step in spliceosome assembly¹¹⁻¹³. Consistent with its central role in spliceosome assembly, U2AF appears to be conserved in higher eukaryotes: immunological and biochemical complementation experiments have identified a likely *Drosophila* homologue of human U2AF (ref. 14). These considerations suggested to us that U2AF might be a target for Sxl-mediated regulation.

FIG. 1 Sxl-regulated alternative splicing of M-tra RNA *in vitro*. **a** (Top), *In vitro* splicing reactions were done in the presence of GST (0.3 $\mu\text{g } \mu\text{l}^{-1}$) or GST-Sxl (0.03, 0.1 and 0.3 $\mu\text{g } \mu\text{l}^{-1}$) and the products analysed by primer-extension using a labelled DNA oligonucleotide complementary to the second exon of the M-tra transcript. M, molecular weight markers (sizes in nucleotides). Bottom, schematic representation of *tra* and M-tra pre-mRNAs. Black boxes: *tra* exons; line: non-sex-specific intron; striped box: female-specific portion of *tra* intron; white boxes: Ad ML sequences. The positions of the 5' and alternative 3' splice sites are indicated together with the sequence of their corresponding polypyrimidine (Py) tracts and AG dinucleotide (in bold). Also indicated is the position of the stop codon (UAG) present in the non-sex-specific open-reading-frame of *tra*. **b**, Primer-extension analysis of the splicing products using splice-junction primers. Left, non-sex-specific splice-junction primer; right, female-specific splice-junction primer. P indicates the position of the primer. Because the 3' end of both oligonucleotides is common, the same length of extension products (72 nucleotides) is expected. **c**, Activity of Sxl without GST moiety. Splicing products of reactions containing either no Sxl (—), or increasing concentrations (0.03, 0.1 and 0.3 $\mu\text{g } \mu\text{l}^{-1}$) of Sxl protein, obtained by thrombin cleavage of the GST-Sxl fusion protein, were analysed as in **b**. Primer-extension bands corresponding to the non-sex-specific and female-specific products were aligned to facilitate comparisons.

METHODS. Total RNA from female *Drosophila melanogaster* flies (Oregon R strain) was reverse-transcribed and PCR amplified²² using oligonucleotides specific for the 5' and 3' ends of Sxl coding regions²³. Sxl cDNA was cloned in pGEX-2T (ref. 24) and Sxl protein was expressed in *E. coli* as a fusion with glutathione-S-transferase and purified as described²⁵. For some experiments, GST-Sxl was digested for 30 min with 0.025 units of thrombin (Sigma) per μg fusion protein, and the reaction stopped with 3 units of hirudin (Boehringer). A fragment of *tra* pre-mRNA (positions 1–530) was obtained from *Drosophila* genomic DNA by PCR amplification²⁶ and cloned into pGEM3 Zf(–) (pGEM-tra). A derivative (pGEM-tra-IE) was prepared by inserting a *Clal-NheI* fragment from pBR322 into the Asp 418 site of pGEM-tra. pGEM-M-tra was obtained by substitution of a *HindIII* fragment from plasmid pMINX²⁷ (containing AdML gene first intron 3' splice site) by a *HindIII* fragment from pGEM-tra-IE containing *tra* alternative 3' splice sites. Linearized pGEM-M-tra was used for *in vitro* transcription with SP6 RNA polymerase²⁸. *In vitro* splicing reactions in HeLa and *Drosophila* nuclear extracts were performed under standard conditions^{29,30}. Primer extension reactions were performed as described²⁹. Second exon oligonucleotide is complementary to *tra* mRNA positions 510–530. The splice-junction-specific oligonucleotides were complementary to the last 5 residues of the first exon and to the first 13 residues of the corresponding second exon.



As a first test of this possibility, we used an RNA mobility shift assay to measure the RNA binding affinities of Sxl and U2AF for the polypyrimidine tract/3' splice region of the *tra* non-sex-specific and female-specific sites. Figure 2a shows that Sxl and GST-Sxl bound to the non-sex-specific polypyrimidine tract with high affinity (apparent $K_d \sim 10^{-9}$ M), whereas binding to the female-specific site was undetectable even at a 60,000-fold higher protein concentration. Figure 2b shows that U2AF also

bound the non-sex-specific site with high affinity (apparent $K_d \sim 10^{-8}$ M). In contrast to Sxl, however, U2AF bound to the female-specific site, although with an affinity 100-fold lower than it did to the non-sex-specific site. A three-nucleotide mutation in the non-sex-specific pyrimidine tract that abolishes Sxl responsiveness *in vivo*⁷ decreased Sxl binding 100-fold and U2AF binding 10-fold.

To investigate further the relative RNA binding specificities

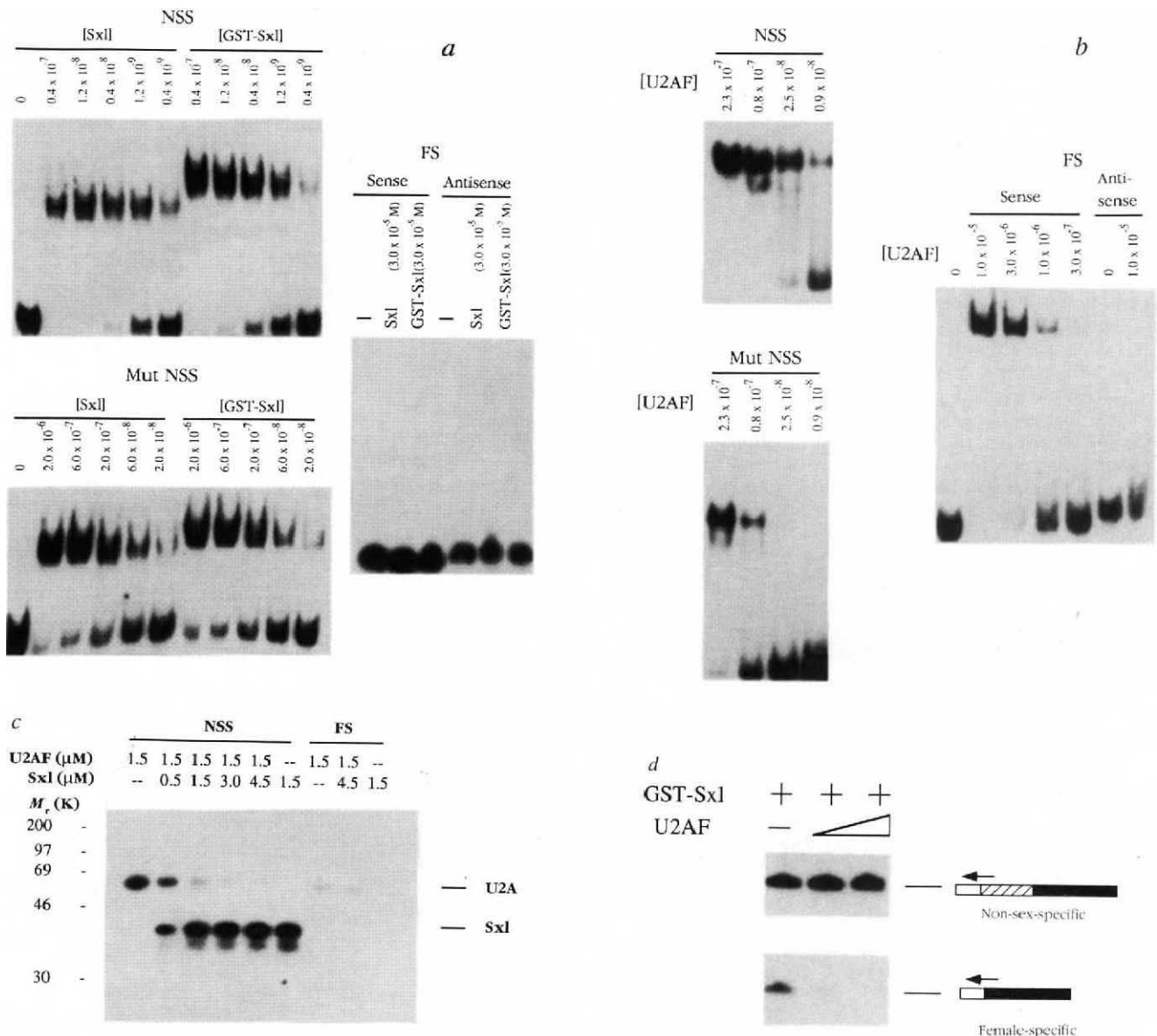


FIG. 2 Binding of Sxl and U2AF to the Py tract/3' splice sites (ss) of *tra*. RNA binding reaction mixtures contained concentrations (molar) of Sxl or GST-Sxl (a), or U2AF (b) proteins indicated above each lane. NSS: RNA probe containing *tra* non-sex-specific Py-tract/3'ss (positions 165–188). mut NSS: mutant version of NSS RNA containing 3 nucleotide substitutions that abolish Sxl response *in vivo*⁷. FS: RNA probe corresponding to *tra* female-specific Py-tract/3'ss (positions 340–363). The apparent K_d values, obtained from the protein concentration that gives 50% binding based on densitometric analysis, for Sxl were: NSS probe, 1.0×10^{-9} M; mut NSS, 1.0×10^{-7} M; FS (sense), $>3.0 \times 10^{-5}$ M; FS (antisense), $>3.0 \times 10^{-5}$ M. The K_d values for U2AF were: NSS probe, 1.5×10^{-8} M; mut NSS, 1.2×10^{-7} M; FS (sense), 2.0×10^{-6} M; FS (antisense), $>10^{-5}$ M. c, Sxl competes with U2AF for binding to the non-sex-specific site. RNA binding reactions containing purified Sxl and/or U2AF, at the concentrations indicated, were UV-irradiated and the crosslinked RNA-protein complexes resolved by SDS-PAGE. On the left are shown positions of protein size markers and on the right, the positions of

U2AF (65K) and Sxl (39K). d, Reversion of Sxl-induced splice site switch by recombinant U2AF. *In vitro* splicing reactions were done in the presence of GST-Sxl ($0.07 \mu\text{g } \mu\text{l}^{-1}$) with or without purified U2AF ($0.07 \mu\text{g } \mu\text{l}^{-1}$ in the second lane, $0.35 \mu\text{g } \mu\text{l}^{-1}$ in the third lane), and the products analysed as in Fig. 1c.

METHODS. U2AF was expressed in bacteria using the T7 RNA polymerase/promoter system³¹, and purified by ion-exchange chromatography³². RNAs corresponding to *tra* non-sex-specific and female-specific 3' splice sites were synthesized as described in ref. 33. Reactions ($20 \mu\text{l}$) containing 10 mM Tris, pH 7.5, 50 mM NaCl (or KCl), 1 mM EDTA, 1 mM DTT, $0.5 \text{ U } \mu\text{l}^{-1}$ RNasin, $0.09 \mu\text{g } \mu\text{l}^{-1}$ BSA, 0.05% glycerol, 3 μg yeast tRNA, appropriate protein dilutions and 1 fmol labelled RNA probe, were incubated on ice for 30 min and resolved on a non-denaturing polyacrylamide gel. Crosslinking with ultraviolet light was as described³², except that no BrdU was used to prepare the RNAs and samples were irradiated at 254 nm.

of Sxl and U2AF, an analysis similar to that shown in Fig. 2 was done using a variety of *Drosophila* and mammalian polypyrimidine tracts/3' splice site regions (Table 1). Only for the non-sex-specific pyrimidine tract/3' splice site of *tra* was the binding affinity of Sxl higher than that of U2AF: in all other cases U2AF affinity was at least 15- to 100-fold higher than that of Sxl.

The RNA binding data shown in Fig. 2 suggested that Sxl could compete with U2AF for binding to the non-sex-specific site. We confirmed this prediction using an ultraviolet crosslinking assay that allows direct visualization of the bound proteins. Figure 2c shows that both U2AF (M_r 65K) and Sxl (39K) were crosslinked to a 32 P-labelled RNA containing the non-sex-specific site polypyrimidine tract. Consistent with the RNA mobility-shift data, U2AF bound to the female-specific polypyrimidine tract with lower efficiency, and there was no detectable crosslinking of Sxl to this site. Most important, as the concentration of Sxl was raised, crosslinking of U2AF to the non-sex-specific polypyrimidine tract decreased while crosslinking of Sxl increased concomitantly.

These results suggested a model in which the splice site switch would depend upon the ratio of Sxl to U2AF. Figure 2d shows that addition of recombinant U2AF to the HeLa nuclear extract counteracted the Sxl-mediated activation of the female-specific 3' splice site. Thus U2AF and Sxl are antagonistic in the regulation of the two alternative 3' splice sites.

U2AF and Sxl are both polypyrimidine-tract-binding proteins. But Sxl represses splicing to the non-sex-specific site and effects splice-site switching, whereas U2AF is a general activator of splicing. We considered that the difference in the activities of these two proteins could be due to an arginine-serine (RS) rich effector domain present in U2AF⁹ but absent from Sxl. A

TABLE 1 Relative binding affinities of Sxl and U2AF for different polypyrimidine tracts/3' splice sites

Organism	Type of intron	Polypyrimidine tract/3' splice sites	Relative affinity Sxl/U2AF
<i>Drosophila</i>	R	<i>tra</i> non-sex-specific	15
<i>Drosophila</i>	R	<i>tra</i> female-specific	<0.06
<i>Drosophila</i>	C	<i>tra</i> intron 2	<0.01
<i>Drosophila</i>	R	P element intron 3	<0.01
Human	C	β -globin intron 1	<0.02
Human	R	Calcitonin intron 3	<0.01

The relative RNA-binding affinities of Sxl and U2AF (the inverse of (K_d Sxl/ K_d U2AF)) are indicated. K_d , dissociation constant determined as for Fig. 2. R, regulated intron; C, constitutively spliced intron. The polypyrimidine tract/3' splice sites examined correspond to the following introns: *tra* non-sex-specific and female-specific: first intron of *transformer* pre-mRNA (Fig. 2); *tra* intron 2: second intron of *transformer* pre-mRNA⁵ (positions 798–821), constitutively spliced in both male and female flies; P element intron 3: third intron of *Drosophila* P element pre-mRNA (position 2,118–2,141), repressed in somatic cell lineage²⁰; β -globin intron 1: first intron of the human β -globin gene pre-mRNA (position 340–373); Calcitonin intron 3: third intron of the human calcitonin gene pre-mRNA (position 1,109–1,132), repressed in cells of neural origin²¹.

prediction of this idea is that if Sxl were provided with the U2AF effector domain, it would fail to mediate splice site switching because it would promote, rather than inhibit, splicing to the non-sex-specific site. To test this hypothesis we constructed a chimaeric protein (GST-USx) containing the effector

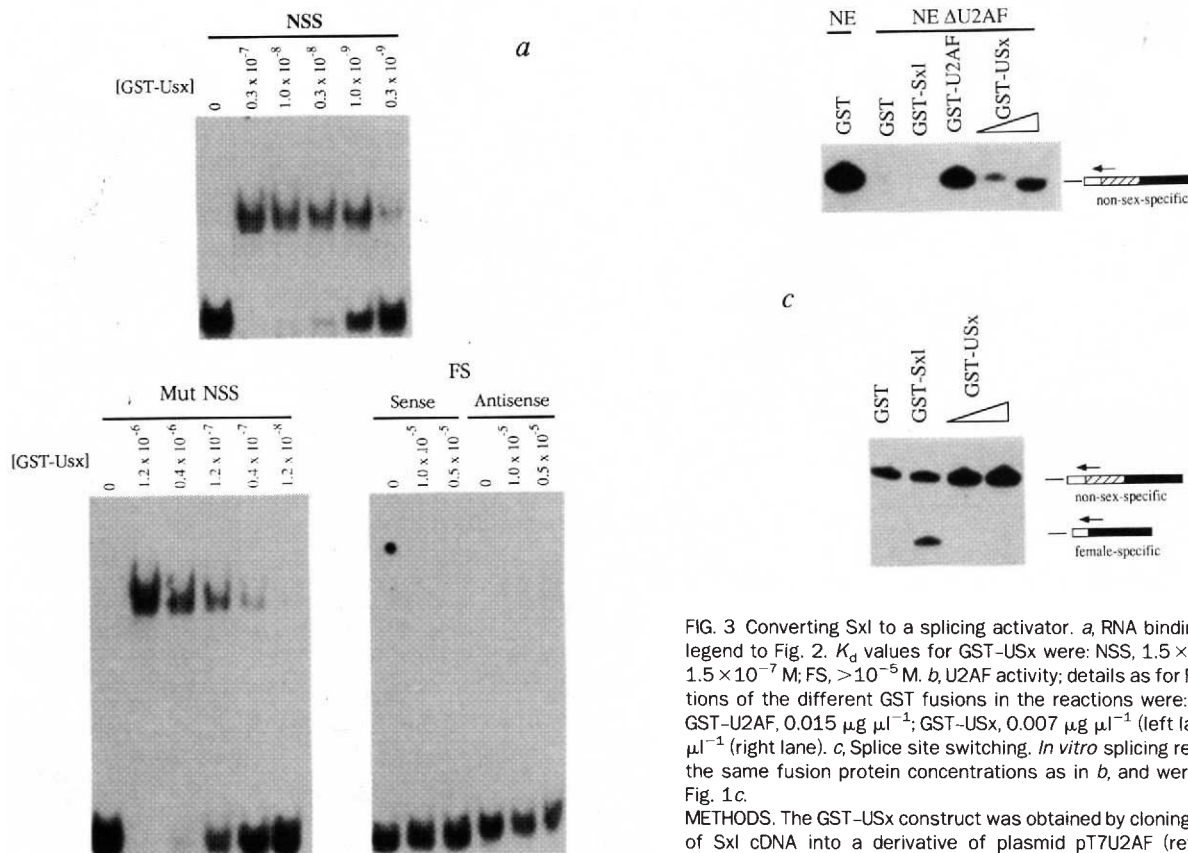


FIG. 3 Converting Sxl to a splicing activator. *a*, RNA binding; for details see legend to Fig. 2. K_d values for GST-USx were: NSS, 1.5×10^{-9} M; mut NSS, 1.5×10^{-7} M; FS, $>10^{-5}$ M. *b*, U2AF activity; details as for Fig. 1c. Concentrations of the different GST fusions in the reactions were: GST, GST-Sxl and GST-U2AF, $0.015 \mu\text{g} \mu\text{l}^{-1}$; GST-USx, $0.007 \mu\text{g} \mu\text{l}^{-1}$ (left lane) and $0.015 \mu\text{g} \mu\text{l}^{-1}$ (right lane). *c*, Splice site switching. *In vitro* splicing reactions contained the same fusion protein concentrations as in *b*, and were analysed as for Fig. 1c.

METHODS. The GST-USx construct was obtained by cloning an *EaeI* fragment of Sxl cDNA into a derivative of plasmid pT7U2AF (ref. 9) in which the sequences corresponding to the two C-terminal RNP-CS of U2AF were deleted by removal of a *HindIII* fragment; this fusion construct was cloned into pGEX-3X (ref. 24) and the GST fusion protein expressed in *E. coli* and purified as described²⁵.

domain of U2AF fused to the complete RNA-binding domain of Sxl.

We assayed GST-USx for three functions: RNA-binding, U2AF activity and splice-site switching. Figure 3a shows that the RNA-binding affinity and specificity of GST-USx is like that of Sxl, and not U2AF. Figure 3b shows that GST-USx has U2AF activity: in a HeLa nuclear extract specifically depleted of U2AF (ref. 14) (NEΔU2AF), GST-USx and GST-U2AF supported splicing to the *tra* non-sex-specific 3' splice site, whereas GST and GST-Sxl did not. Figure 3c shows that GST-USx failed to promote splice-site switching: whereas GST-Sxl activated the female-specific site, GST-USx only increased use of the non-sex-specific site.

A model for Sxl-mediated regulation of *tra* pre-mRNA splicing must answer three questions. First, why in the absence of Sxl is the non-sex-specific 3' splice site exclusively used? Second, what accounts for the specificity of Sxl action? Third, what is the molecular basis for switching between the two alternative 3' splice sites? Below we describe a model for Sxl action that addresses these issues.

The exclusive use of the non-sex-specific 3' splice site in the absence of Sxl can be explained by the 100-fold higher affinity of U2AF for this site relative to the female-specific site. The higher uridine content of the non-sex-specific polypyrimidine tract (Fig. 1a, bottom) most probably accounts for this difference in U2AF binding affinity (see ref. 9).

Our RNA binding data also explain why Sxl regulates *tra* alternative splicing without affecting the splicing of most other pre-mRNAs. We propose that the specificity of the splice site switch reflects the relative RNA-binding specificities of the repressor Sxl and the activator U2AF. U2AF binds to a variety of polypyrimidine tracts⁹, as would be expected for a general splicing factor. In contrast, Sxl binds with high affinity only to the *tra* non-sex-specific polypyrimidine tract (Fig. 2 and Table 1), as would be expected for a splicing regulator.

We have presented four lines of evidence indicating that U2AF is antagonized by Sxl at the non-sex-specific site, and we propose that this is the basis for the splice-site switch. First, the relative RNA-binding affinities of U2AF and Sxl are consistent with this proposal (Fig. 2a, b). Second, Sxl competes with U2AF for binding to the non-sex-specific, but not to the female-specific polypyrimidine tract (Fig. 2c). Third, the relative use of the two 3' splice sites depends on the Sxl/U2AF ratio (Fig. 2d). Finally, fusion of the U2AF effector domain to Sxl prevents splice-site switching (Fig. 3c): if another splicing component were antagonized by Sxl, addition of the U2AF effector domain should not relieve the Sxl-mediated inhibition.

In addition to *tra*, other steps in *Drosophila* sexual determination also involve 3' splice site regulation¹. For example, Sxl regulates the splicing of its own pre-mRNA. Compared to *tra* pre-mRNA splicing, however, the regulated splicing of Sxl pre-mRNA appears to occur by a more complex mechanism that requires additional components^{15,16}. *Tra* and *Tra-2* activate a female-specific 3' splice site in *doublesex* pre-mRNA by a positive mechanism¹⁷⁻¹⁹. It will be important to determine whether these other alternative splicing events are also regulated by controlling the accessibility of U2AF to specific polypyrimidine tracts. □

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Cloning and expression of human TAF_{II}250: a TBP-associated factor implicated in cell-cycle regulation

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BASAL transcription by human RNA polymerase II requires the coordinate action of several ancillary factors (TFIIA-J)¹ and can be regulated by various promoter-specific DNA binding proteins. An additional class of factors, called coactivators, are dispensable for basal transcription but are indispensable for regulation by transcriptional activators²⁻⁴. Biochemical studies established that some coactivators are associated with the TATA-binding protein (TBP) to form the TFIID complex³⁻⁶. We therefore set out to define the relationship between TBP and these TBP-associated factors (TAFs). Here we describe the cloning, expression and properties of the first human TAF, hTAF_{II}250. The hTAF_{II}250 gene is identical to a gene, *CCGI* (refs 7, 8), implicated in cell-cycle progression. Recombinant hTAF_{II}250 binds directly to TBP both *in vitro* and in yeast, and participates in the formation of the TFIID complex. This largest TAF may therefore play a central role in TFIID assembly by interacting with both TBP and other TAFs, as well as serving to link the control of transcription to the cell cycle.

The hTFIID complex can be isolated by affinity chromatography using antibodies specific to hTBP (refs 4, 5). The purified complex contains at least seven distinct TAFs which range in *M_r* from 30-250K and which copurify with hTBP (Fig. 1a, lane 2). Of these, the 250K subunit binds hTBP directly, as determined by protein blotting analysis (far-western analysis; Fig. 1a, lane 3). Using affinity-purified TAFs to immunize mice, we generated polyclonal and monoclonal antibodies crossreacting with different TAFs (Fig. 1b) and used them to screen λgt11 expression complementary DNA libraries. Several clones were isolated, including λH1 (Fig. 2b), which contains a 1.1-kb insert. To determine which, if any, TAF is encoded by λH1, we expressed this cDNA as a glutathione-S-transferase (GST) fusion protein, purified the tagged protein by glutathione-affinity chromatography, and raised antibodies against it. Antisera directed against GST-λH1 specifically crossreacted with the 250K

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TAF (Fig. 2a), indicating that λ H1 contains part of the gene encoding hTAF_{II}250. The DNA sequence of λ H1 contains an open reading frame identical to that of a human gene implicated in cell-cycle regulation, called *CCG1* (refs 7, 8). Expression of *CCG1* is necessary to overcome arrest in G1 when the temperature-sensitive mutant hamster cell line, ts13, is held at the non-permissive temperature^{7,9}.

As λ H1 only encodes a small portion of hTAF_{II}250 (Fig. 2b), we isolated several additional clones from a primary HeLa cDNA library, including λ H2, which contained a 5.3-kb insert (Fig. 2b). Interestingly, several other cDNAs encoding this protein contained an internal insertion (hTAF_{II}250) or deletion (hTAF_{II}250 Δ) when compared to the *CCG1* sequence (Fig. 2b), implying that multiple hTAF_{II}250 proteins may be generated by alternative splicing of a primary transcript. As the predominant RNA species isolated by polymerase chain reaction (PCR) from HeLa cells encodes 21 additional amino acids between *CCG1* residues 177 and 178 (Fig. 2b), we used this version in further studies.

Although the finding that a cDNA isolated by antibodies directed against TAFs encodes a cell-cycle gene is exciting, it was important to provide some functional evidence that this clone indeed encodes a *bona fide* TAF, which is a subunit of hTFIID. As shown in Fig. 1a, hTAF_{II}250 forms part of the purified hTFIID complex and interacts directly with hTBP. To show that the cDNA we had isolated truly encoded hTAF_{II}250, we therefore sought to show that recombinant hTAF_{II}250 participates in the formation of the hTFIID complex and binds hTBP.

To demonstrate that the recombinant hTAF_{II}250 expressed from a vaccinia virus vector becomes associated with endo-

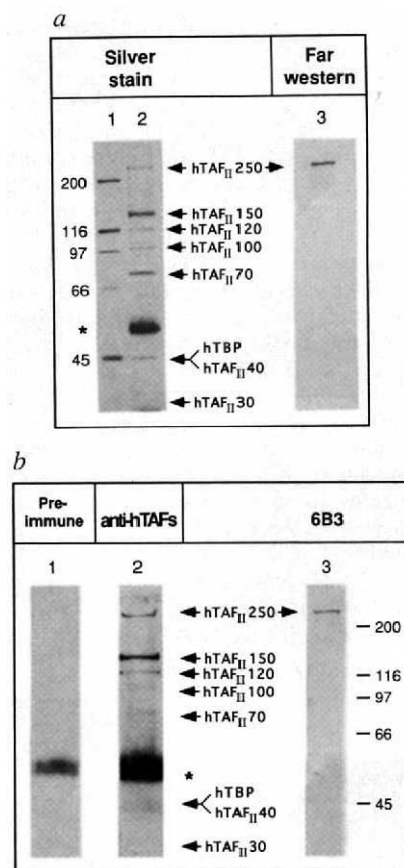
genous hTFIID in HeLa cells, we engineered a version containing a haemagglutinin antigen (HA) epitope at the N terminus of hTAF_{II}250. Antibodies against hTBP (Fig. 3a, lanes 1, 2, 7 and 8), hTAF_{II}150 (Fig. 3a, 1A5; lanes 3 and 4), or hTAF_{II}100 (Fig. 3a, 6B1; lanes 5 and 6) were used to immunopurify the hTFIID complex from HeLa cells infected with either recombinant or control vaccinia virus. The immunopurified complexes were separated by gel electrophoresis and analysed by western blotting using either a monoclonal antibody directed against the HA-tag in the virally encoded hTAF_{II}250, or a monoclonal antibody, 6B3, raised against endogenous hTAF_{II}250. The anti-HA antibody crossreacted with a 250K protein in the hTFIID complex prepared from HeLa cells infected with recombinant but not control virus (Fig. 3a, lanes 1–6), whereas 6B3 recognized hTAF_{II}250 in both extracts (Fig. 3a, lanes 7 and 8). Recombinant hTAF_{II}250 thus associates with hTBP *in vivo* and forms part of the hTFIID complex.

To test for a direct interaction between recombinant hTAF_{II}250 and hTBP, we performed a far-western analysis with radiolabelled hTBP and immunopurified HA-tagged hTAF_{II}250 immobilized on nitrocellulose membrane. The full-length hTAF_{II}250 (Fig. 3b, lanes 2 and 4) bound hTBP even in the absence of other TAFs or coactivators. The independently isolated *Drosophila* homologue of hTAF_{II}250 also interacts with TBP and can bind at least one additional TAF (R. Weinzierl *et al.*, unpublished results). This largest TAF may thus be responsible for the initial assembly of the TFIID complex by binding directly to TBP and other TAFs.

To analyse the interaction between hTAF_{II}250 and hTBP more precisely, we used the two hybrid system in yeast cells¹⁰. Yeast expressing hybrid constructs encoding both hTAF_{II}250 fused to

FIG. 1 Immunoaffinity-purified hTFIID complex: interaction with hTBP and production of hTAFs specific antibodies. *a*, The largest subunit of human TFIID complex interacts directly with hTBP. Partially purified hTFIID fraction was incubated with affinity-purified anti-hTBP polyclonal antibody and protein A-Sepharose (Pharmacia). The immunoprecipitated proteins were subjected to 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and detected by silver staining (lane 2). In addition to hTBP, the human TFIID complex consists of at least 7 components: hTAF_{II}250, hTAF_{II}150, hTAF_{II}120, hTAF_{II}100, hTAF_{II}75, hTAF_{II}40 (comigrating with hTBP), and hTAF_{II}30 (more prominent on other gels). Molecular weights were estimated on the basis of their migration relative to standards in SDS-polyacrylamide gels. Immunoprecipitated TAFs were used for far-western analysis¹⁴ with ³⁵S-methionine labelled hTBP (lane 3). The molecular masses (in K) of protein standards (BioRad) are shown in lane 1. Asterisk indicates IgG. *b*, Specificity of polyclonal and monoclonal antibodies against human TAFs. Immunoprecipitation reactions, as shown in *a*, lane 2, were analysed by western blotting. Antibodies tested included the pre-immune (lane 1) or immune serum obtained from a mouse immunized with immunopurified hTFIID complex (anti-hTAFs, lane 2), and a monoclonal antibody, 6B3, specific for the 250K subunit of hTFIID (lane 3). The positions of hTAFs (lane 2) and *M_s* (K) of protein standards (BioRad) are indicated. Asterisk indicates IgG.

METHODS. *a*, Immunoprecipitation was modified from ref. 4. Affinity-purified anti-hTBP antibody (0.5 μ g) was added to 200 μ g hTFIID (phosphocellulose, 0.48–1.0 M KCl) fraction, and the mixture nutated for 2–4 h at 4 °C. Protein A-Sepharose was then added and nutation continued for an additional 2–4 h. Antibody-antigen complexes were pelleted by low-speed centrifugation, washed four times with 0.1 M KCl-HEMG (25 mM HEPES, 12.5 mM MgCl₂, 0.1 mM EDTA, 20% glycerol) containing 0.1% NP40 and 1 mM DTT. The immunoprecipitated hTFIID complex was subjected to 8% SDS-PAGE and silver-stained. For far-western analysis¹⁴, proteins were blotted onto nitrocellulose membrane and hybridized with ³⁵S-labelled hTBP. The ³⁵S-labelled hTBP was produced by *in vitro* transcription/translation from pTβhTBP as described¹⁵. *b*, hTFIID complex used to immunize mice for antibody production was prepared as follows. Immunoprecipitated hTFIID complex purified from 250I HeLa cells was eluted from the protein A-Sepharose-antibody complex with 0.1 M KCl-HEMG containing 1 M guanidine-HCl, 0.1% NP40 and 1 mM DTT. Under these conditions, TBP remained bound to the antibody. Eluted TAFs were dialysed against 0.1 M KCl-HEMG containing 0.1% NP40 and 1 mM DTT. The mixture of proteins containing ~1–2 μ g of each TAF was used to immunize a mouse¹⁶. The immune response was monitored by western blot analysis using the anti-



hTAF polyclonal serum. After a series of five boosts, the mouse was killed and the spleen used for production of monoclonal antibody-producing hybridoma cell lines. Identification of hybridoma cell lines producing hTAF-specific antibodies was by western blot analysis of immunopurified hTAFs.

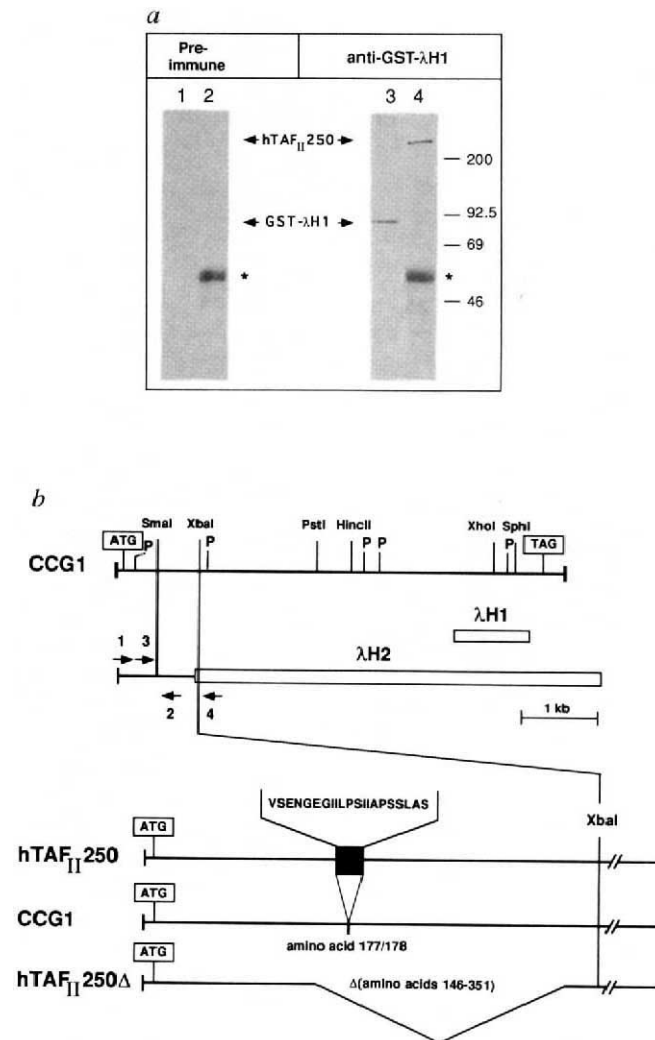
the DNA binding domain of GAL4 and hTBP fused to the activation domain of GAL4 (Fig. 4) produced high levels of β -galactosidase, showing that the hTAF_{II}250: hTBP interaction was sufficiently selective and efficient to allow increased transcription of a lacZ reporter construct containing GAL4 binding sites (Fig. 4). Interestingly, hTAF_{II}250 also interacted, although less efficiently, with a truncated version of human TBP containing only the conserved C-terminal 180 amino acids (chTBP), but not with a construct containing the 'species-specific' N-terminal domain of human TBP (nhTBP). These results are in agreement with far-western experiments using radiolabelled chTBP and nhTBP as probes (S.R., unpublished) and indicate that residues 160 to 339 on the outer surface of TBP¹¹ may be responsible for hTAF_{II}250 binding.

Our unexpected finding that the genes for hTAF_{II}250 and CCG1 are identical suggests an intriguing link between a subunit of hTFIID and the expression of genes involved in the control of the cell cycle. Interestingly, CCG1 is a nuclear phosphoprotein containing a putative HMG-box and a proline-rich cluster⁸, domains thought to mediate DNA binding and transcriptional activation, respectively, in other transcription factors.

On the basis of these motifs, it was suggested that CCG1 might be a sequence-specific transcription factor regulating genes involved in the progression through G1 (ref. 8). It now seems clear, however, that CCG1/hTAF_{II}250 is part of the hTFIID complex and not a promoter-specific transcription factor. Although not fully understood, the ts13 mutant phenotype may therefore result from a general decrease of transcription which can be restored by overexpression of CCG1/hTAF_{II}250. Alternatively, it may reside in CCG1/hTAF_{II}250 itself, leading to a defective TFIID complex unable to mediate activation by a subset of transcription factors responsible for cell-cycle-specific gene activation¹². Once hTAF_{II}250 forms a complex with hTBP, some portion of this large protein, such as the putative HMG-box, may contact DNA, perhaps downstream of the transcription initiation site so as to generate the characteristic extended DNase I footprint seen when TFIID is fully bound¹³. The availability of both recombinant hTAF_{II}250 and the ts13 mutant cell line offers a unique opportunity to study hTFIID complex assembly and function, and may also allow us to address the mechanisms of cell-cycle-specific gene activation. □

FIG. 2 Cloning of the 250K subunit of hTFIID complex and identification as CCG1. **a**, Antisera against GST- λ H1 fusion protein specifically recognizes the 250K subunit of hTFIID complex. Pre-immune (lanes 1 and 2) and anti-GST- λ H1 immune (lanes 3 and 4) sera were used to probe blots containing purified GST- λ H1 protein (lanes 1 and 3) and immunoprecipitated hTFIID complex (lanes 2 and 4). M_r markers (Amersham) are indicated. Asterisk indicates IgG. **b**, Cloning of hTAF_{II}250 reveals identity to CCG1. The top line shows schematically the CCG1 cDNA⁸ with several restriction sites that were used to characterize hTAF_{II}250 cDNA clones. The predicted translation start codon (ATG), translation termination codon (TAG) and *Pvu*II restriction sites (P) are indicated. Positions of λ phages λ H1 and λ H2 with respect to CCG1 cDNA are indicated by open boxes. The 5' end of hTAF_{II}250 was obtained by two PCRs using the primers 1+2 and 3+4, indicated by arrows. Below is a more detailed diagram of the 5' region of hTAF_{II}250, CCG1 and hTAF_{II}250 Δ cDNAs. The black box in hTAF_{II}250 cDNA indicates the 63-bp insertion encoding an additional 21 amino acids between residues 177/178 of CCG1. The hTAF_{II}250 Δ cDNA contains a 618-bp in-frame deletion resulting in loss of residues 146–351 with respect to CCG1 or 146–372 with respect to hTAF_{II}250.

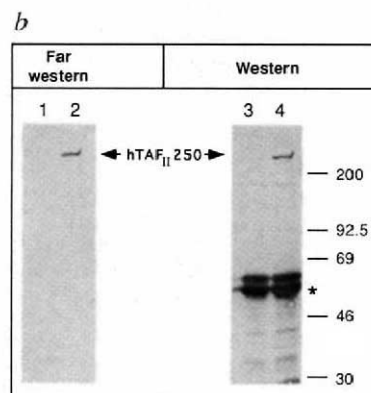
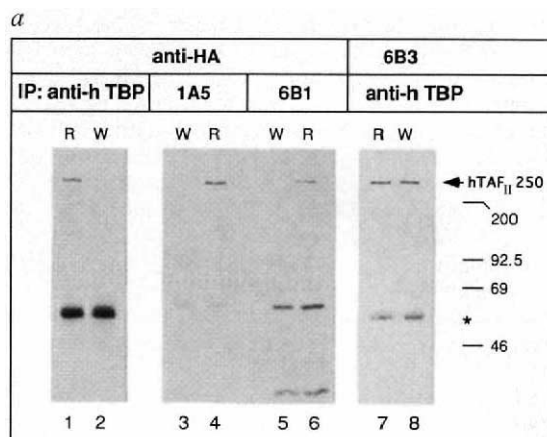
METHODS. **a**, An expression screen of 2.4×10^6 PFU from a λ gt11 HeLa S3 cDNA library (Clontech) was carried out using the anti-hTAFs polyclonal serum described in Fig. 1b. 38 primary signals were identified, of which 6 were plaque-purified. λ phage DNA was prepared and analysed by *Eco*RI restriction enzyme digestion. λ H1 contained a 1.1-kb insert which was subcloned into the *Eco*RI site of pGEX1 (Pharmacia) to express a GST- λ H1 fusion protein. The resulting construct was transformed into *Escherichia coli* TG2, and following induction with 0.5 mM isopropyl- β -D-thiogalactoside (IPTG) the induced protein was purified on glutathione-Sepharose 4B beads (Pharmacia). 2 μ g (per injection) of the fusion protein was used to immunize a mouse. Test bleeds were taken and used for western blotting. **b**, Poly(A)⁺ RNA from HeLa cells was used for construction of a directional cDNA library in λ ZAPII (Stratagene) as described.¹⁷ Using a randomly ³²P-labelled probe derived from the λ H1 cDNA insert, 15 independent cDNA clones were isolated from 1.2×10^6 original PFU. The cDNA inserts were rescued by the zapping procedure¹⁸ and characterized extensively by restriction enzyme analysis and Southern blotting. The longest cDNA clone, pH2, isolated from λ H2, contains a 5.3-kb insert, revealing an extended 3' untranslated region but missing about 1.15 kb of 5' sequences compared with CCG1. This 5' region was generated by PCR using HeLa poly(A)⁺ RNA as described¹⁷. Two sets of PCR primers were designed according to the CCG1 cDNA sequence⁸: PCR-I, forward primer 1: 5'-TATTTCCGGCATATGGGACCCGGCTG-3' (position 40 to 65, containing an engineered *Nde*I restriction site at the translation start codon), and reverse primer 2: 5'-GAAGTCCACTTCTCACCAG-3' (position 578 to 597). PCR-II, forward primer 3: 5'-TACCAGCAGCATATGGGG-AGCTTGACAG-3' (position 421 to 447) and reverse primer 4: 5'-GCTCTAA-GGAAGCCAGCCTGCCAGGCTTG-3' (position 1,343 to 1,371). All PCR products were subcloned into pBluescript KS (Stratagene) and sequenced. The most abundant product of PCR-II, a 1-kb fragment, included a 63-bp in-frame insertion, whereas a minor 330-bp fragment revealed a 618-bp in-frame deletion with respect to the CCG1 cDNA. To generate a full-length hTAF_{II}250



cDNA, the product of PCR-I and the 1-kb PCR-II product were joined via the shared *Sma*I restriction site. Subsequently the 1.2-kb *Xba*I fragment of the resulting plasmid was cloned into *Xba*I-digested pH2 to generate the full-length cDNA clone pH2AF_{II}250. The sequence of this clone was in agreement with the CCG1 cDNA sequence⁸.

FIG. 3 Analysis of hTAF_{II}250 and hTBP interaction. **a**, Recombinant hTAF_{II}250 assembles into hTFIID complex. The hTFIID complex was immunoprecipitated from nuclear extracts prepared from HeLa cells infected with either recombinant HA-tagged hTAF_{II}250 (R; lanes 1, 4, 6 and 7) or wild-type virus (W; lanes 2, 3, 4 and 8). For immunoprecipitation (IP) the following antibodies were used: polyclonal anti-hTBP, monoclonal antibody 1A5, raised against endogenous hTAF_{II}150, and monoclonal antibody 6B1, raised against endogenous hTAF_{II}100. Immunoprecipitated hTFIID was separated by SDS-PAGE and analysed by western blotting using either the anti-HA antibody¹⁹ (BABC0, lanes 1–6) or the monoclonal antibody 6B3 (lanes 7 and 8). Asterisk indicates IgG. **b**, Direct binding of ³⁵S-labelled hTBP to recombinant hTAF_{II}250. Recombinant HA-tagged hTAF_{II}250 was expressed in SF9 cells using a baculovirus expression system (Pharmingen). Whole cell extracts prepared from uninfected SF9 cells (lanes 1 and 3) or from SF9 cells infected with recombinant baculovirus (lanes 2 and 4) were immunoprecipitated with an anti-HA monoclonal antibody, subjected to 8% SDS-PAGE, transferred to a nitrocellulose membrane and incubated with either ³⁵S-labelled hTBP (lanes 1 and 2) or the monoclonal antibody 6B3 (lanes 3 and 4). The positions and molecular weights of protein standards (BioRad) are indicated.

METHODS. **a**, To construct an HA-tagged version of hTAF_{II}250, we generated a plasmid, pSK-HAX, containing the haemagglutinin antigen epitope¹⁹, factor-X cleavage site, and in-frame *NdeI* cloning site. A 6.3-kb *NdeI*/*Asp*718 fragment from pTAF_{II}250 was inserted into pSK-HAX to generate pHAX-hTAF_{II}250. A 6.0-kb *SpeI* fragment thereof containing the complete coding region of hTAF_{II}250, was inserted into the *XbaI* site of the vaccinia virus expression vector pAbT4537 (ref. 20) (Applied bioTechnology). Generation of the recombinant virus and viral infection were performed as described²¹. Extracts from HeLa cells infected with recombinant virus, vTAF_{II}250, or control virus (New York City Board of Health strain of vaccinia virus) were fractionated by phosphocellulose chromatography as described⁴. hTFIID complexes from the 0.48–1.0 M KCl fraction were immunoprecipitated with either affinity-purified anti-hTBP, 1A5 (anti-hTAF_{II}150) or 6B1 (anti-hTAF_{II}100) antibodies, subjected to 8% SDS-PAGE and analysed by western blotting. Monoclonal antibodies 1A5 and 6B1 immunoprecipitated hTFIID complexes as efficiently as anti-hTBP (E.H.W., unpublished). hTFIID complexes immunoprecipitated with 1A5 and 6B1 were eluted as described in Fig. 1. **b**, To generate an HA-tagged version of hTAF_{II}250 in the baculovirus expression system, we first generated a new baculovirus vector, pVL1392HAX, derived from pVL1392 (Pharmingen). This vector contains the HA antigen epitope¹⁹, factor X cleavage site, and unique in-frame *NcoI* and *NdeI* restriction sites. A 6.0-kb *NdeI*/*SpeI* fragment from pTAF_{II}250 was inserted into pVL1392HAX, creating pBAX-hTAF_{II}250. Whole cell extracts from either SF9 cells or SF9 cells infected with recombinant baculovirus were prepared in 0.4 M KCl-HEMG (including 0.04% NP40, 1 mM DTT, 0.2 mM



AEBSF (4-(2-aminoethyl)-benzenesulphonylfluoride), 0.1 mM sodium meta-bisulphite) by sonication and used directly for immunoprecipitation with the anti-HA antibody as described for Fig. 1. The precipitate was subjected to 8% SDS-PAGE and blotted onto nitrocellulose membrane. The filter was probed first with ³⁵S-labelled hTBP, as described in Fig. 1 legend, and subsequently with monoclonal antibody 6B3.

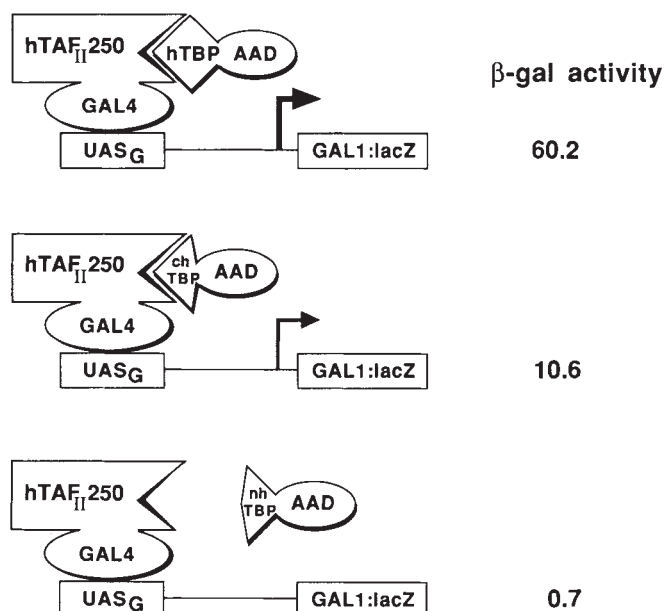


FIG. 4 hTAF_{II}250 interacts with the conserved domain of hTBP. Full-length hTAF_{II}250 was expressed as a fusion protein with the DNA binding domain of GAL4. Full-length hTBP, the conserved C-terminal region of hTBP (chTBP) and the evolutionarily diverged N-terminal region of hTBP (nhTBP) were expressed as fusion proteins with the GAL4 acidic activation domain (AAD). The corresponding plasmids were transformed into yeast bearing an integrated GAL1:lacZ fusion under control of a promoter bearing GAL4 binding sites (UAS_G). The resulting β -galactosidase activity is given as β -galactosidase units per mg protein. When tested with GAL4 (1–147) alone, hTBP, chTBP and nhTBP fusion proteins gave 0.18, 0.27 and 0.4 units of β -galactosidase activity, respectively. GAL4/hTAF_{II}250 alone produced only 0.14 units of activity.

METHODS. hTAF_{II}250, fused to the DNA binding domain of GAL4 (residues 1–147), was constructed by inserting a cDNA fragment coding for full-length hTAF_{II}250 into the pAS1 vector (provided by S. Elledge). The activation domain fusions were obtained by cloning inserts into the pGAD1F vector²². Hybrid proteins generated included the acidic activation domain of GAL4 (residues 768–881) fused to either full-length hTBP, residues 160–339 of hTBP, or residues 1–159 of hTBP. The constructs described were transformed into the yeast strain Y153 (a, gal4, gal180, his3, trp1-901, ade2-101, ura3-52, leu2-3, 112, URA3:Gal1:lacZ, LYS2:Gal-His3; provided by S. Elledge) and β -galactosidase assayed as described⁶.

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The p250 subunit of native TATA box-binding factor TFIID is the cell-cycle regulatory protein CCG1

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THE protein TFIID is a general transcription factor¹ which initiates preinitiation complex assembly^{2–4} through direct interaction with the TATA promoter element^{5,6}. It is a multisubunit complex containing a small TATA-binding polypeptide (TBP) and other TBP-associated factors (TAFs) ranging in size from about 30–250K (refs 7–10). Although native TFIID can mediate both activator-independent (basal) and activator-dependent transcription in reconstituted systems^{3,5,6}, TBP itself can mediate only basal transcription^{11,12}, even in cases where TBP or the general factor TFIIB are known to interact directly with transcriptional activators^{13–15}. TFIID subunits other than TBP must therefore be essential cofactors, and thus potential targets for activators, consistent with earlier demonstrations that activators interact with TFIID (refs 3, 5, 16, 17). Here we show that the 250K subunit of TFIID is identical to a gene product previously implicated in progression through the late G1 phase of the cell cycle^{18,19}. Part of p250 may thus serve a specific function in the activation of a subset of genes important for cell cycle progression.

TFIID affinity-purified with anti-TBP antibody showed TBP-associated components with M_r s of ~250, 150, 120, 100, 80, 75, 65 and 60K, as previously described⁹. Microsequence analysis of three peptides derived from the 250K component showed perfect matches with three regions in the human CCG1 gene

product (Fig. 1a), a nuclear DNA-binding protein known to be important for cell cycle progression^{18,19}. Given this indication of identity between p250 and CCG1, the CCG1 sequence was used to isolate overlapping complementary DNAs and to construct a full-length cDNA expression vector (Fig. 1 legend).

To confirm that p250 is identical to CCG1, we prepared antibodies against a synthetic peptide containing residues 103–123 of CCG1. In an immunoblot analysis of samples separated by SDS-PAGE (Fig. 1b), this antibody crossreacted with a polypeptide of the same size as the largest TFIID subunit in both partially purified TFIID (phosphocellulose fraction; lane 3) and anti-TBP affinity-purified TFIID (lane 4). The antibody also crossreacted with a polypeptide of this size obtained by baculovirus expression of the CCG1 cDNA (lane 1) but not a control (β -galactosidase) cDNA (lane 2).

Far-western (protein blot) analysis indicates that the largest subunit of affinity-purified TFIID (p250) interacts directly with TBP⁹. To confirm the equivalence of CCG1 and p250, the CCG1 and TFIID preparations already analysed by immunoblot were separated by SDS-PAGE, blotted, and probed with radiolabelled TBP (Fig. 1c). The TBP bound to a 250K polypeptide in the baculovirus-expressed CCG1 preparation (lane 1), the partially purified TFIID (lane 3), and the affinity-purified TFIID (lane 4), but not in the control baculovirus-expressed protein preparation (lane 2). The band of 200K in the baculovirus CCG1 preparation is apparently derived from the 250K protein, and is only apparent at high levels of expression. Its constant reactivity with both anti-CCG1 antibody and TBP relative to p250 provides additional evidence for the specificity of these reagents.

Although our inability to reconstitute a native TFIID from isolated components has precluded functional analysis of the expressed CCG1 protein, our results clearly indicate that the CCG1 protein and the 250K subunit of TFIID are identical. Our recent success in cloning a cDNA encoding the largest subunit of *Drosophila* TFIID based on direct peptide sequence analysis has confirmed this conclusion. The sequence of the 230K *Drosophila* TFIID subunit is 50% identical to that of human p250/CCG1, indicating that the proteins are homologous (T. Kokubo *et al.*, unpublished observations).

The sequence of the p250 cDNA described here exactly matches that of CCG1 (refs 18, 19). Thirty-one per cent of the 1,872 residues in the protein (calculated $M_r = 212,666$) are charged, giving it a highly hydrophilic and presumably flexible structure. Several putative structural motifs in p250/CCG1 have previously been noted. These and other features are summarized in Fig. 2.

Like TBP and TFIIB (refs 20, 21), p250/CCG1 contains a direct repeat of, in this case, 132 residues. Interestingly, embedded within each repeat is a region termed the 'bromodomain', found in a variety of global or pleiotropic transcription factors^{22–24}. Although its function is unknown, it has been suggested that the amphipathic helices in bromodomains may be involved in protein-protein interactions²³. If so, the intramolecular symmetry of the domains in p250/CCG1 would facilitate interactions with other symmetrical molecules such as TBP and DNA.

As noted previously¹⁹, p250/CCG1 also contains an HMG box, a DNA-binding domain of about 80 residues which has been identified in several classes of proteins. HMG boxes can be divided into three classes on the basis of their sequence and function^{25,26}. The p250/CCG1 sequence does not fit clearly into any of these groups, however, and may therefore have a specialized function.

Other features of p250/CCG1 include (1) several regions with potentially significant sequence similarities to the conserved 2.1, 4.1 and 4.2 subregions of bacterial σ -factors²⁷, some of which occur in other general initiation factors²¹; (2) a region of 183 residues which is 21% identical (and 36% similar) to the yeast protein SW14, a cell cycle-regulated DNA-binding protein involved in the activation of other cell cycle-regulated genes (such as HO endonuclease and G1 cyclins) in late G1 phase²⁸; and (3) a region of 180 residues which is 22% identical (and 39%

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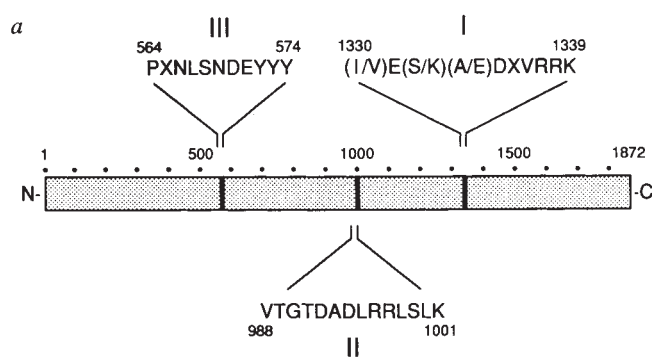
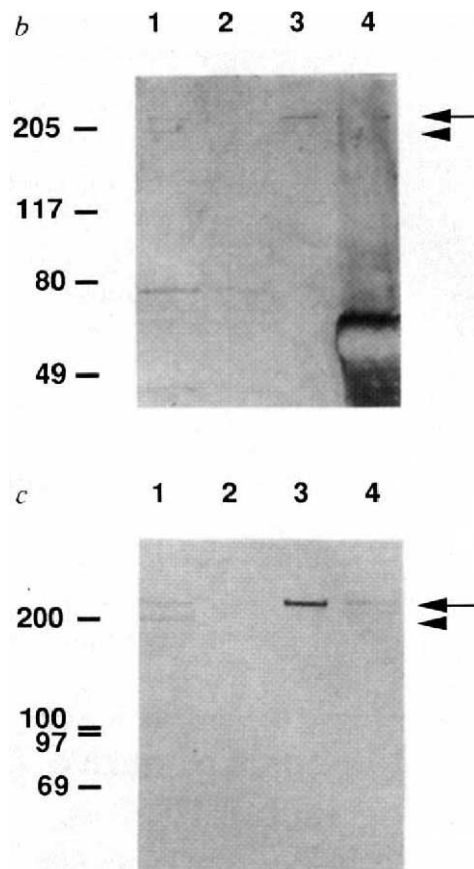


FIG. 1. Confirmation that p250 and CCG1 are identical, and interact with TBP. *a*, Positions of sequenced p250 peptides in CCG1. I, II and III indicate the amino-acid sequences determined from the p250-derived peptides. Uncertain residues are indicated in parentheses or by an X. The corresponding amino-acid sequences predicted from CCG1 (ref. 19) are: I, IESADEVRRK; II, VTGTDADLRRLSLK; III, PXNLSNDEYYY. *b*, Immunoblot analysis of natural TFIID and recombinant CCG1 preparations. After SDS-PAGE and transfer, samples were probed with anti-CCG1 peptide antibody. Arrow indicates the position of p250/CCG1. The band indicated by an arrowhead is probably a partial degradation product of p250/CCG1 whose amount varies in different preparations. Lane 1, lysate from Sf9 cells infected with recombinant baculovirus expressing CCG1. Lane 2, lysate from Sf9 cells infected with recombinant baculovirus expressing β -galactosidase (Invitrogen). Lane 3, phosphocellulose 0.85 M KCl TFIID fraction⁹. Lane 4, TBP-associated polypeptides from affinity-purified TFIID⁹. The migration positions of standard molecular weight markers are shown in the left column. *c*, Direct interaction of TBP with recombinant CCG1. After resolution by SDS-PAGE, electrotransfer to nitrocellulose membranes and renaturation, samples were probed with ³⁵S-labelled TBP. The arrow indicates the position of p250/CCG1; the arrowhead indicates the apparent degradation product described in Fig. 1*b* and the text. Lane 1, lysate from Sf9 cells infected with recombinant baculovirus expressing CCG1. Lane 2, lysate from Sf9 cells infected with recombinant baculovirus expressing β -galactosidase. Lane 3, phosphocellulose 0.85 M KCl TFIID fraction. Lane 4, TBP-associated polypeptides from affinity purified TFIID. Positions of labelled standard molecular weight markers (Amersham) are shown in the left column.

METHODS. TFIID from HeLa nuclear extract-derived phosphocellulose 0.85 M KCl fractions was affinity-purified with anti-TBP antibody as described⁹. After elution from immobilized antibody columns and trichloroacetic acid precipitation, the TBP-derived polypeptides (TAFs) from multiple preparations were resolved by SDS-PAGE (7% acrylamide) and electrophoretically transferred to polyvinylidene difluoride membrane (PVDF). After staining the membrane with Ponceau S, the clear band corresponding to p250 (estimated as more than 10 pmol) was excised and digested with endoproteinase Lys-C on the membrane, and the products were resolved by reverse-phase HPLC and subjected to microsequence analysis. To obtain a full-length cDNA, human Namalwa, placenta, hepatoma and kidney (Clontech) cDNA libraries were screened with oligonucleotides based on the p250-derived peptide and published²¹ CCG1 DNA sequences. From these, five overlapping clones covering DNA position 231 through the poly(A) sequences (encoding all but the first 78 amino acids) were selected for further use. For the N-terminal region, the 234-bp open reading frame was constructed with synthetic oligonucleotides whose sequence around the first methionine, 5'-CCGGTCTATGGAC-3' (initiation ATG is underlined), was replaced by 5'-TCGACATATGGAC-3' to create *Sal*I and *Nde*I restriction sites. The synthetic N terminus and the five overlapping cDNA clones were ligated, subcloned in pBlueScriptIIKS (Stratagene) and confirmed by DNA sequencing. To construct a plasmid for expressing a recombinant protein in baculovirus,



the CCG1 cDNA was cut with *Sal*I, partially filled in with Klenow enzyme plus dGTP and dATP, cut with *Bam*HI within the multiple cloning site, and gel-purified. pBlueBacHisB (Invitrogen) was digested with *Bam*HI, partially filled in with Klenow plus dGTP and dATP, and further cut with *Bgl*II. The vector and insert were ligated to produce pBlueBacHis250 and the correct in-frame ligation was confirmed by sequencing. CCG1 in this construct contains an extra 35-amino-acid tag containing 6 consecutive histidines. Recombinant baculovirus was produced following Invitrogen's instructions. For expression of CCG1, Sf9 cells (Invitrogen) were infected with viruses expressing either CCG1 or β -galactosidase and collected at 60 hours post-infection. An amount of extract from $2-4 \times 10^5$ cells was used in each lane for SDS-PAGE. For the phosphocellulose TFIID and affinity-purified TFIID preparations, the amounts of protein per lane were about 20 μ g and 5 ng (excluding antibody), respectively. Immunoblot assays were done as described⁹, except that anti-CCG1 peptide antiserum was used as primary antibody at 1:125 dilution. The antibody was prepared by injecting a rabbit with the peptide STEDAVDYSDINEVAEDESRR, conjugated with BSA. Far-western blot analysis was done essentially as described⁹. The samples analysed were the same as those used for *b*.

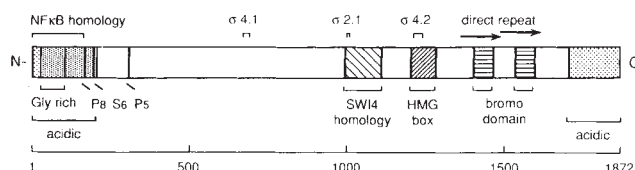


FIG. 2. Structural motifs in p250/CCG1. Positions of several structural motifs are indicated (see also ref. 19). The numbers indicate amino-acid residue positions. P8, S6 and P5 indicate stretches of 8 prolines, 6 serines and 5 prolines, respectively; acidic domains indicate a 204-residue N-terminal region with a net negative charge of -34 and a 185-residue C-terminal domain with a net negative charge of -53. Gly-rich indicates a region (residues 32-99) with 22% glycine residues. The NF- κ B homology extends from position 1-177 and shares sequence similarity (see text) with residues 733-900 of p100/NF- κ B (ref. 29). The SW14 homology extends from position 966-1,146 and shares sequence similarity (see text) with residues 728-911 of SW14 (ref. 28). The HMG box extends from residues 1,197-1,276 and shares sequence similarity with many other HMG boxes^{19,25,26}. The direct repeats containing the bromodomains²²⁻²⁴ encompass residues 1,365-1,495 and 1,486-1,618. The sigma homology regions indicate residues of 671-686 (σ 4.1), residues 1,000-1,010 (σ 2.1) and residues 1,211-1,239 (σ 4.2).

similar) to a C-terminal region in the presumptive precursor of an alternative DNA-binding subunit forming part of the transcription factor NF- κ B (ref. 29).

As reported previously¹⁹, p250/CCG1 also contains multiple consensus target sequences for casein kinase II, cyclic AMP-

dependent protein kinase, and Ca^{2+} /calmodulin-dependent kinases, and is a substrate for casein kinase II *in vitro*. Given its ability to interact directly with TBP, its sheer size suggests that it may play a major role both in recruiting other TAFs to the TFIID complex and in receiving signals from various tran-

scriptional regulators. Although a function extrinsic to TFIID cannot be excluded, the co-fractionation of all detectable p250/CCG1 with TFIID (data not shown) argues that it subserves a major cell cycle function as part of TFIID.

Only some G1 transcription functions are disrupted in ts cell mutants that can be complemented by wild-type p250/CCG1 (ts13 and tsBN462), and the cells survive for some time at the

restrictive temperature^{18,19,30}. The transcription defect in these cells thus appears to be restricted to only some genes, possibly those controlled by transcription factors with a particular type of activation domain. Such a domain might interact with a region of p250/CCG1 not otherwise used as an activator target (perhaps modified at certain stages of the cell cycle), so affording an additional checkpoint for cell cycle progression. □

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Yeast tRNA^{Asp} recognition by its cognate class II aminoacyl-tRNA synthetase

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AMINOACYL-RNA synthetases can be divided into two classes according to structural features inferred from sequence alignments^{1–3}. This classification correlates almost perfectly with the attachment of the amino acid to the 2'-OH (class I) or 3'-OH (class II) group of the terminal adenosine^{4–6}. Six subgroups of higher homology can be inferred from sequence analysis^{7,8}. The five aminoacyl-tRNA synthetases whose crystal structures are known (MetRS, TyrRS and GlnRS in class I, SerRS and AspRS in class II)^{9–13} belong to different subgroups. Two of them, GlnRS and AspRS, have been cocrystallized with their cognate tRNA^{11,13}. AspRS, like six other members of class II, is an α_2 dimer. Yeast tRNA^{Asp} exhibits five identity determinants: the three anticodon bases, the discriminator base G73 and the base pair G10–U25¹⁴. We report here that the refined crystal structure of AspRS complexed with tRNA^{Asp} at 2.9 Å resolution reveals three regions of contact, each involving a domain of AspRS and at least one identity determinant of tRNA^{Asp}. The mode of binding of the acceptor stem of tRNA^{Asp} by AspRS can be generalized to class II aminoacyl-tRNA synthetases, whereas the deciphering of the anticodon, which involves a large conformational change of the loop and the formation of a bulge, is more specific to the aspartic system.

Although the aspartyl-tRNA synthetase (AspRS) dimer has a rather compact shape, each subunit is very elongated and shows two distinct domains connected by a small hinge module (Fig. 1). The C-terminal domain (residues 241–557) is the largest and contains the active site. The three conserved motifs of class II synthetases are found in this region¹. The N-terminal domain

is involved in the recognition of the anticodon of the tRNA. The dimeric enzyme binds two tRNA molecules symmetrically but with some differences at the active site (Table 1). Apart for one interaction between Lys 293, which is part of the first conserved motif, and the phosphate group of U1, all direct contacts formed by one tRNA molecule involve the same synthetase monomer (Table 1). The total buried surface or contact area of one tRNA molecule is 2,500 Å². This represents about 20% of the solvent-accessible surface of the free tRNA and is similar to what was observed in the glutamyl system¹¹. The N-terminal domain makes few interactions with the C-terminal domain of the same monomer but forms an important interface with the C-terminal domain of the other subunit. Thus, information may be transferred in a cooperative manner from the N-terminal domain of one subunit to the active site of the other subunit.

The main feature of the C-terminal domain is the active-site cavity formed by a six-stranded antiparallel β -sheet and partly closed by loops and α -helices¹³. This region contains the three signature motifs of class II aminoacyl-tRNA synthetases (aaRS), which interact with the acceptor stem of the tRNA and perform the catalysis (Fig. 2a). Motifs 1 and 2 participate in the positioning of the acceptor stem and motif 2 and 3 bind the ATP. This suggests that the mechanism involved in the recognition of this part of the tRNA can probably be generalized to most if not all class II synthetases. Two loops clamp the acceptor stem of the tRNA. The variable loop of motif 2 (residues 327–334) approaches the tRNA molecule on the major groove side and interacts with the discriminator base G73 and the first base pair of the stem. Another segment (residues 423–428) interacts with the phosphate groups of A72 and G73. This peptide is part of a domain (residues 373–438) of variable size within the aaRS inserted after motif 2.

The hinge connecting the C-terminal and N-terminal domains of the protein is a distinct globular module of four short helices (residues 207–240). It interacts with the ribose and phosphate groups of U11 and U12 rather than with the determinant G10–U25 (Fig. 2b and table 1). The molecular mechanism by which the determinant acts is not protein-mediated: mutations of G10 or U25 are likely to disrupt the tertiary interaction formed with G45 and thus lead to a conformational change in this part of

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the tRNA. This agrees with the observation that such mutations affect the Michaelis constant, K_M , in contrast with mutations at other determinants, which affect the catalysis constant K_{cat} (ref. 14).

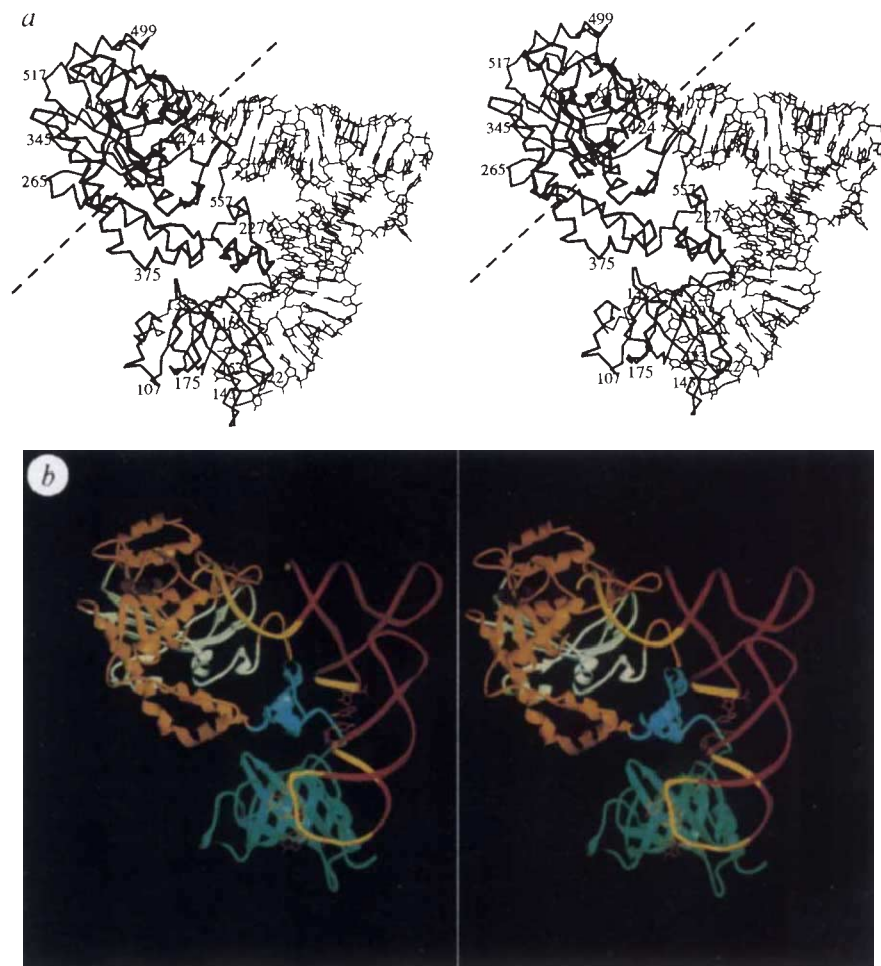
The N-terminal domain is built around a five-stranded β -barrel. The domain approaches the anticodon loop of the tRNA on the side of the major groove (Fig. 3a). If the strands are numbered in the order of the sequence, the topological succession is S1 S4 S5 S3 S2 S1. Successive strands are antiparallel, except S5 and S3. An α -helix is inserted between strands S3 and S4. The same topological organization has been found in completely different enzymes, namely staphylococcal nuclease¹⁵, heat-labile enterotoxin¹⁶ and verotoxin-1¹⁷ (A. Murzin, personal communication). In all these enzymes, this region is involved in the binding of either nucleotides or oligosaccharides. The interaction with the anticodon loop is specific to the AspRS system, but sequence analyses have shown that the principles can probably be generalized to the subgroup comprising AspRS, AsnRS and LysRS¹⁸⁻²⁰. Because no significant homology with other aaRS could be detected, the N-terminal domain may be characteristic of the subgroup specificity. In this domain, 5 of the 14 amino acids involved in direct contacts with tRNA are conserved both in prokaryotic and eukaryotic AspRS, and in the subgroup of AspRS, AsnRS and LysRS from *Escherichia coli*. Three of the residues (Phe 127, Gln 138 and Arg 119) recognize the base U35 which is conserved in the cognate tRNAs of the members of the subgroup. Arg 119 also interacts with the ribose of residue C36. The fourth, Glu 188,

which in the yeast AspRS system binds G34, can interact equally well with the modified guanine present in the tRNA^{Asp} and tRNA^{Asn} from different organisms. In tRNA^{Lys}, the nucleotide in position 34 is cytidine, uridine or one of their derivatives. Note that Lys 142, which also binds G34 of yeast tRNA^{Asp}, is not present in *E. coli* to make space for the bulky base Q (queuosine). The fifth conserved residue, Gly 122, plays a pivotal role at the end of the β -sheet where any side chain would interfere with the base at position 32 in the anticodon stem.

The three bases of the anticodon are protruding out to maximize contacts with the protein, which illustrates the flexibility of the tRNA molecule (Fig. 3b). When compared to the situation in the free state^{21,22}, the conformation of tRNA^{Asp} in the complex is characterized by the closing of the two limbs. The regular RNA A-form double helix of the acceptor stem is not altered, but the anticodon stem is smoothly bent inward starting with base pair G27-C43. The most striking feature is that the anticodon loop (bases ψ 32 to C38) has unravelled so that five bases protrude outward. The backbone forms a bulge at the modified purine 37 (m1G37) which is stabilized by two intramolecular hydrogen bonds, one between the O2' of C38 and the phosphate of C36, and the other between the N2 of m1G37 and the phosphate of U25.

The crystal structure of yeast tRNA^{Asp}-AspRS complex confirms the modular character of the members of the synthetase family and clarifies the role of each structural component. The manner in which the active site domain binds the CCA end of tRNA^{Asp} and ATP can probably be considered a model of class

FIG. 1 a, General view of the complex. One monomer of AspRS (C α backbone) and one molecule of tRNA^{Asp} are shown. The position of the local twofold axis is indicated. This and the other black-and-white stereopictures were made using the program MOLSCRIPT²⁵. The first 65 residues of each monomer of AspRS are at least partly disordered in the crystal and could not be traced in the maps. They are not essential because it has been shown that the first 75 residues can be removed by mild digestion without affecting the enzymatic activity of the protein²⁶. Crystals belong to space group P2₁2₁2 with unit cell parameters $a=210.2$ Å, $b=146.2$ Å, $c=86.1$ Å and with one dimeric molecule per asymmetric unit. The crystal structure was solved by multiple isomorphous replacement with three derivatives and the phases were improved by solvent flattening and noncrystallographic symmetry averaging. The model was refined through energy minimization with X-PLOR²⁷. The R -factor is 23.1% for 49,370 reflections (90% of possible data, $F/\sigma(F)>3$) in the 7-2.9 Å resolution range without including any solvent molecules. The r.m.s. bond deviation from ideality is 0.010 Å and r.m.s. angle deviation is 1.8°. The mean B -factor is 30 Å². b, Regions of interaction between AspRS and tRNA^{Asp} (program RIBBONS²⁸). The contact regions of the tRNA are in yellow, with the identity determinants shown. The C-terminal domain of AspRS is in brown, with class II signature motifs in white. The N-terminal domain is in green and the junction peptide in blue.



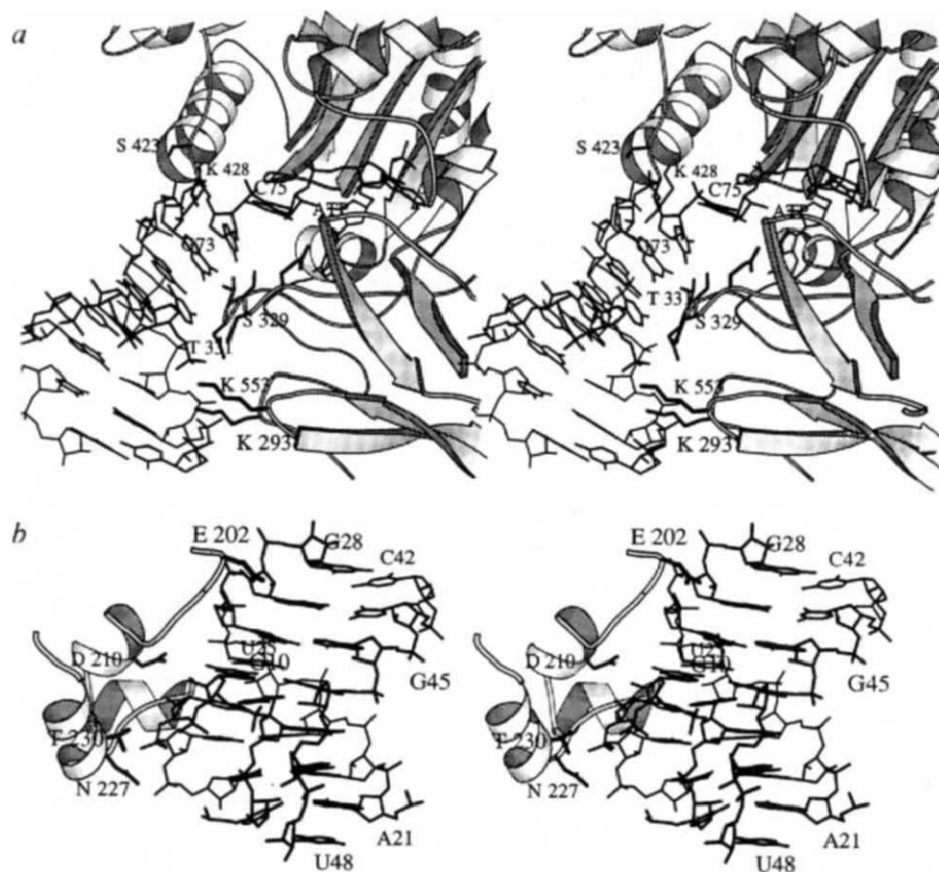


FIG. 2 *a*, Contacts in the region of the acceptor stem of tRNA^{Asp} . Residues 327–331, which form the variable part of the second conserved motif of class II synthetases, make base-specific hydrogen bonds with the discriminator base G73 and the first base pair U1–A72. Ser 423, Thr 424 and Lys 428 bind to the phosphate groups of A72 and G73. The phosphate group of U1 is responsible for the only interaction with the other synthetase monomer, through Lys 293. *b*, Contacts in the region of the D-stem of tRNA^{Asp} . The ribose-phosphate backbone from U11 to U12 is secured by a number of interactions with protein residues Glu 202, Asp 210, Asn 227 and Thr 230. This is the region forming the hinge between the C-terminal and the N-terminal domains. None of these interactions is base-specific, and none involves the determinant base pair G10–U25.

FIG. 3 *a*, Contacts in the region of the anticodon of tRNA^{Asp} . Lys 155, a strictly conserved residue in all known AspRS, binds to the phosphate groups of G30, an interaction which may be important for the correct relative positioning of the protein and the anticodon stem. The guanine G34 forms two hydrogen bonds with the conserved Glu 188 and interacts with Lys 142 through N7, in agreement with protection experiments toward dimethylsulphate done in solution²⁹. U35 is sandwiched between C36 and the strictly conserved Phe 127, and forms a hydrogen bond with the conserved Gln 138. C36 is hydrogen bonded to main chain groups of Pro 178 and Lys 180; its ribose binds to the side chain of the conserved Arg 119. *b*, Superposition of the anticodon stem and loop in the free (thin line) and complexed (thick line) tRNA^{Asp} . In the complex, the canonical U-turn conformation is disrupted after $\Psi 32$. $\Psi 32$ and C38 form an additional distorted non-Watson-Crick base pair (one hydrogen bond between $\Psi 32$ O4 and C38 N4) stacked over C31–G39. U33 points toward the solvent with a weak connection to the enzyme. The stacking of the anticodon bases observed in free tRNA^{Asp} is totally unravelled. G34 makes no contact with U35 which is imperfectly stacked to C36 while being associated with Phe 127.

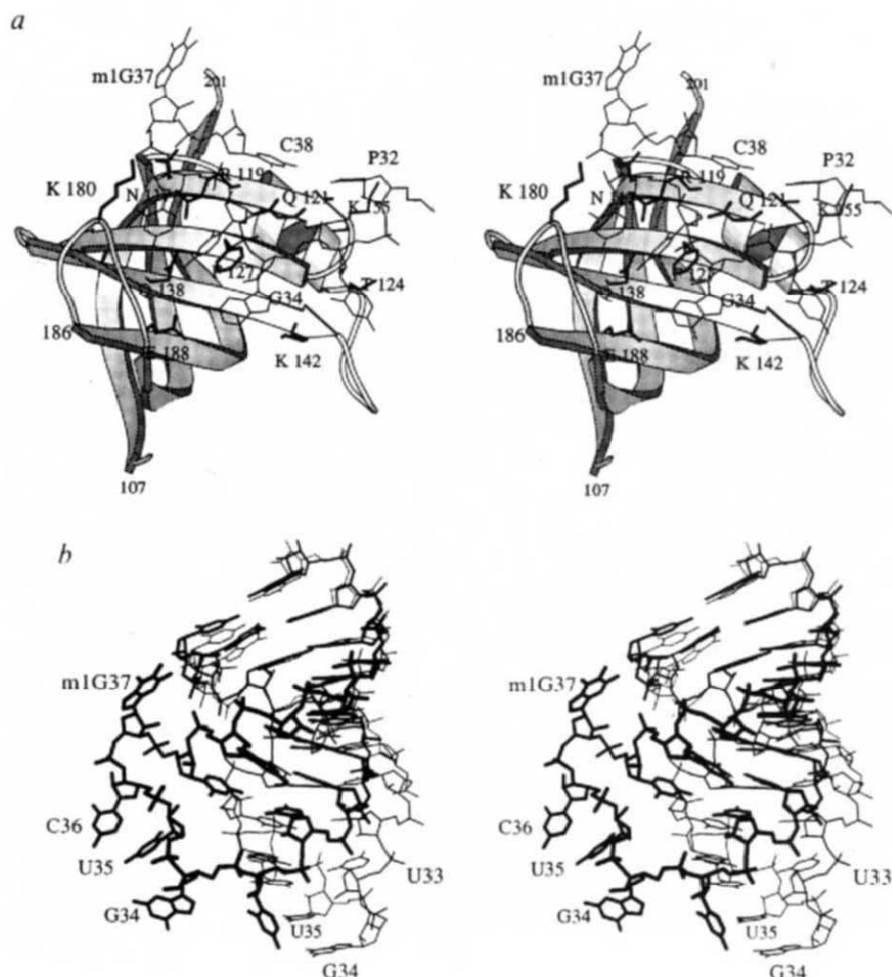


TABLE 1 tRNA synthetase interactions (distance <3.5 Å)

tRNA		AspRS	
U1	OP	Lys 293*	Nζ
	OP	Asn 328	Nδ
	O4	Asn 330	N
A72	OP	Lys 428	Nζ
	N6	Asn 330	O
G73	OP	<i>Thr 424</i>	Oγ
	OP	<i>Ser 423</i>	Oγ
	O6†	<i>Thr 331</i>	Oγ
	N1†	<i>Ser 329</i>	Oγ
	N2†	Asn 328	O
C74	N2†	Glu 327	Oε
	N3†	<i>Ser 329</i>	Oγ
	N4†	<i>His 334</i>	Nε
C67	OP	<i>Lys 553</i>	Nζ
U11	O2'	<i>Asp 210</i>	Oδ
U12	OP	<i>Thr 230</i>	Oγ
	OP	Asn 227	N
G27	O2'	Glu 202	Oε
G30	OP	Lys 155	Nζ
Ψ32	N3	Gln 120	O
U33	O4	<i>Thr 124</i>	Oγ
G34	N7	Lys 142	Nζ
	N2	Glu 188	Oε
U35	O4'	Gln 121	Nε
	N3	Gln 138	Oε
	O2	Arg 119	Nη
	—	Phe 127	Stacking
C36	O4'	Arg 119	Nη
	O2	<i>Ser 181</i>	Oγ
	O2	Lys 180	N
	N4	Pro 178	O
m1G37	OP	Lys 180	Nζ
C38	OP	Asn 117	Nδ
	N4	Gln 120	O
	N3	Gln 121	Oε

Interactions within the active site (nucleotides C75 and A76) are not included. Residues in bold are highly conserved in all AspRS, whereas those in italics are conserved in eukaryotes only. In tRNA, phosphate oxygens are named OP, atoms with primes belong to the ribose, all others to the base.

* This residue belongs to the other subunit.

† The conformations are different in the two subunits. In the map of the binary complex the active sites are different in the two subunits. In subunit B, although the crystals were grown in the absence of ATP in the mother liquor, a clear electron density is visible in which an AMP model can be fitted. In the other subunit, this site is partially occupied by the terminal adenosine of tRNA as a result of a conformational change which extends up to the first base pair of the acceptor stem. In subunit B, where the CCA end of tRNA occupies its functional location, A76 is stacked on Phe 304 with its ribose group above and close to the β phosphate of ATP. The ATP binding site essentially comprises the class II conserved Glu 327 and Phe 338 (stacked on the purine ring) and the glycine-rich strand of motif 3 (Gly 526–Gly 528) which interacts with the ribose-phosphate groups. The invariant residues of motifs 2 and 3 bind to the α-phosphate (Arg 325) and to the ribose (Arg 531). Addition of ATP to the crystals leads to fully symmetrical dimers. A detailed analysis of the structure–function relationship in the active site will be published (J.C. *et al.*, manuscript in preparation).

II aaRS. Subgroup specificity is then obtained by the addition of a module at the N-terminal end. Another module, not conserved in prokaryotic AspRS, is inserted after the conserved motif 2 of class II and plays an important role in the specific recognition of tRNA. An additional recognition element interacting with the minor groove of the acceptor stem may be present in some class II enzymes, for example in the *E. coli* AlaRS system where the main tRNA identity determinant is the third base pair of the acceptor stem^{23,24}. The insertion peptide after motif 2 could also act as a minor groove recognition module. □

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CORRECTION

Lead isotope evidence for young trace element enrichment in the oceanic upper mantle

Alex N. Halliday, Gareth R. Davies, Der-Chuen Lee, Simone Tommasini, Cassi R. Paslick, J. Godfrey Fitton & Dodie E. James

Nature **359**, 623–627 (1992)

WE have been unable to precisely reproduce some of the lead isotope data for Madeira lavas reported in the above paper. In the light of further analyses the correct compositions are as follows:

Sample number	²⁰⁶ Pb/ ²⁰⁴ Pb	²⁰⁷ Pb/ ²⁰⁴ Pb	²⁰⁸ Pb/ ²⁰⁴ Pb
MD4	19.159	15.533	38.825
MD10	19.182	15.535	38.864
MD32	19.097	15.559	38.946
MD40	19.143	15.573	38.864
MD64	19.038	15.556	38.753

The ²⁰⁷Pb/²⁰⁴Pb, ⁸⁷Sr/⁸⁶Sr and ¹⁴³Nd/¹⁴⁴Nd ratios still plot within the field of Atlantic MORB, whereas the ²⁰⁶Pb/²⁰⁴Pb and ²⁰⁸Pb/²⁰⁴Pb ratios are relatively high for MORB. Therefore, although the revised Pb isotopic compositions plot closer to other OIB, the overall interpretation is unchanged. □

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Isolating RNA from whole blood — the dawn of RNA-based diagnosis?

Donald E. Macfarlane and Christopher E. Dahle

A novel cationic surfactant solution is used to lyse blood cells and precipitate RNA and DNA. The RNA is recovered by extracting the pellet with a small volume of guanidinium isothiocyanate or formamide.

THE addition of a novel cationic surfactant (Catrimox-14) solution to whole blood results in lysis of the cells and the precipitation of RNA and DNA complexed with the surfactant. After centrifugation, the RNA can be recovered from the pellet by either extraction with hot buffered formamide followed by ethanol precipitation, or with guanidinium isothiocyanate followed by extraction at slightly acid pH and isopropanol precipitation. This simple and rapid method yields RNA that is suitable for analysis by reverse transcriptase and the polymerase chain reaction (RT-PCR) without further purification. Compared with methods that require prior isolation of leukocytes or pathogens from blood, isolating RNA from blood directly increases the feasibility of using RNA-based diagnosis of infections, inflammatory diseases, leukaemia and inherited disorders.

DNA is not an ideal target for molecular diagnosis: most eukaryotic DNA consists of non-informative sequences between genes, and of introns within them. Compared with DNA, analysing RNA can be more informative because the level of expression of many genes reveals the activity of the cell as well as its genetic origin, and because the cell usually contains multiple copies of RNA sequences of interest. The utility of RNA-based diagnosis has been restricted by the difficulty in extracting RNA from clinical specimens before the RNA is degraded.

Recent advances in the analysis of RNA, particularly the use of RT-PCR¹, have greatly increased the information that can be derived from small amounts of RNA even when it is partially degraded. Traditional northern blots are performed with about 10 µg of undegraded RNA, preferably using RNA enriched with mRNA. RT-PCR can detect a few hundred copies of a sequence of RNA, and typical analyses can be performed with much less than 1 µg of cellular RNA.

Widely used methods of extracting RNA from cells depend on the ability of chaotropic concentrations of guanidinium isothiocyanate (often with an anionic surfactant) to lyse the cells and simultaneously inhibit RNase. This results in a complex solution of DNA, RNA, lipid, small molecules, and protein. The RNA can be recovered from this mixture by ultracentrifugation, or the

DNA, protein, and most other molecules can be removed by phenol extraction at slightly acid pH, followed by precipitating the RNA with an alcohol². Although such methods can be applied to whole blood, researchers studying the RNA of blood leukocytes or blood-borne pathogens have generally preferred to use centrifugal procedures to isolate the cells or pathogens from the blood sample before extracting the RNA. This may be to avoid the inhibitors of PCR found in blood (such as porphyrins and haematin³). Unfortunately, it is often not practical to perform cell separating procedures in a timely fashion in a clinical environment.

Because some species of RNA (especially those encoded by some oncogenes and cytokines) have a short half-life in the cell, there are advantages in mixing the blood sample with an RNA extractant as soon as it is drawn. The extractant must be capable of inhibiting RNase, as blood contains enough RNase to destroy extracellular RNA in a few seconds⁴.

Cationic surfactants

The ability of cationic surfactants to precipitate RNA and DNA was reported a generation ago⁵⁻⁷. This precipitation is probably due to the formation of electrically

TABLE 1a Pellet size after lysis of blood

	12-TMA	14-TMA	16-TMA	12-BA	14-BA	16-BA	18-BA
Bromide	2	2	2				
Chloride	1	2	1	2	2	(2)	(5)
Phosphate	2	2	1	2	1	(1)	(1)
Sulphate	0	1	1	2	2	2	(2)
Formate	3	0	2	1	0	(0)	1
Acetate	0	0	1	0	0	(0)	1
Propionate	3	0	2	1	0	0	0
Oxalate	1	1	1	3	3	3	(2)
Malonate	0	1	1	3	3	2	(2)
Succinate	1	1	1	3	3	(2)	(2)
Citrate		3	3				

TABLE 1b Precipitation of RNA (per cent)

	12-TMA	14-TMA	16-TMA	12-BA	14-BA	16-BA	18-BA
Bromide	91	88	79				
Chloride	95	96	70	77	78	68	57
Phosphate	94	95	87	86	86	94	88
Sulphate	101	102	100	76	82	83	87
Formate	44	28	80	33	40	94	86
Acetate	15	30	32	33	31	90	76
Propionate	68	24	82	24	30	66	70
Oxalate	102	99	101	85	75	72	68
Malonate	102	92	93	95	94	80	87
Succinate	100	91	91	93	90	82	89

METHODS. Blood (200 µl, anticoagulated with citrate), was added to 1 ml of the indicated surfactant (0.1 M), together with 50 µl phosphate buffered saline containing 10 µg tRNA as carrier and 20,000 c.p.m. ³²P-RNA (a 2,000-base transcript). One hour later, the mixture was centrifuged, and the supernatant was aspirated and discarded. The pellet was examined visually and graded (Table 1a) as to its size and colour as follows: 0 = pellet almost invisible to the unaided eye; 1 = lightly coloured pellet or smear of material on side of tube with minimal volume; 2 = brown pellet 2–3 mm in long axis, incompletely covering the bottom of the tube; 3 = brown pellet 3–4 mm in long axis, completely covering bottom of tube; 4 = dark brown, 4–5 mm in

long axis; 5 = greater than 5 mm in long axis. Some surfactant solutions tended to crystallize on storage at room temperature. In these cases, the results are given in parentheses. The pellet was then dissolved with 100 µl formamide containing 0.3 M sodium acetate and 50 mM acetic acid by heating and vortexing. The radioactivity was then estimated by scintillation counting, and is expressed as a percentage of the added radioactivity (Table 1b). The abbreviations are 12-, 14-, or 16-TMA: acyl-trimethylammonium with acyl of 12, 14, or 16 carbons in length, and 12-, 14-, 16-, or 18-BA: acyl-benzyltrimethylammonium with acyl of 12, 14, 16, or 18 carbons in length.

neutral complexes between the (polyanionic) nucleic acid and the surfactant, leaving the hydrophobic tail of the surfactant facing the bulk phase⁸. These complexes are soluble in polar solvents, and they are dissociated by the addition of a salt. We reported that cationic surfactants could lyse cells, rendering their proteins soluble⁹, and that the direct addition of cationic surfactants to cells could be used to isolate the RNA and DNA¹⁰.

In attempting to apply this method to whole blood, we found that commercially

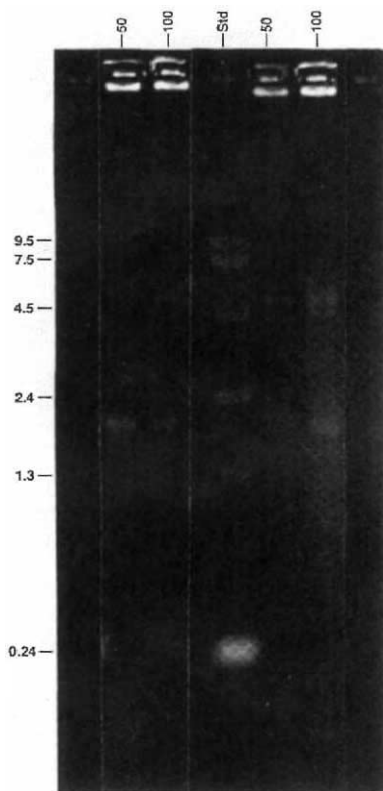


FIG. 1 Isolation of RNA from whole blood. Fifty or 100 µl blood anticoagulated with 1/10 vol. 3.2% sodium citrate was added to 1 ml 0.1 M Catrimox-14 surfactant and 0.1 M DTT and mixed. Fifteen minutes later, the mixture was centrifuged (16,000g, 5 min), and the supernatant was aspirated and discarded. The pellet was washed with 1 ml RNase free water, and dissolved in 100 µl 4 M guanidinium isothiocyanate, 0.2 M sodium acetate pH4 with occasional vortexing for 10 min. One hundred microlitres of phenol:chloroform:isoamyl alcohol pH 5.2; (25:24:1; Amresco, Solon Ohio) was then added and vortexed. After centrifugation (16,000 g, 2 min), the upper aqueous layer was added to 100 µl isopropanol, and the RNA was allowed to precipitate at -20 °C for 30 min. After centrifugation, the supernatant was discarded, the pellet was washed with 70% ice-cold ethanol, and dried briefly *in vacuo*. It was redissolved in a formaldehyde buffer and electrophoresed on a denaturing 1.2% agarose gel (65 × 100 mm), and stained with ethidium bromide. The figure is a photograph of the resulting gel under ultraviolet light. The size markers (stds: Gibco BRL, Gaithersburg, Maryland) are indicated in bases.

available cationic surfactants were unable to lyse blood cells completely, and that they incompletely precipitated RNA from dilute solutions.

To overcome these inadequacies, we synthesized and tested several novel cationic surfactant solutions, finding (to our surprise) that the nature of the counter ion had a decisive influence on both blood lysis and RNA precipitation. Table 1a shows the size of the pellet obtained after centrifuging a mixture of blood and a series of test surfactants. Table 1b shows the ability of the surfactants to precipitate radioactive RNA from dilute solutions. Examination of these results shows that solutions prepared by neutralizing alkyltrimethylammonium hydroxides with dicarboxylic acids were capable both of lysing the blood well, and almost quantitatively precipitating the RNA.

Within limits, the length of the surfactant tail (the major determinant of the critical micellar concentration) had little influence on the ability of a cationic surfactant salt to precipitate RNA. This may be because (in concentrated aqueous mixtures), the surfactant exists in an equilibrium between one or more ordered structures in which the hydrophobic tails are juxtaposed (micelles, tubes, sheets, complexes with protein or with nucleic acids), and (as a minor component) surfactant monomer¹¹. Increasing the length of the hydrophobic tail increases the stability of each of the ordered structures to the same extent, resulting in a decrease in the monomer concentration, but no change in the relative concentration of the ordered structures.

With the exception of the complex with nucleic acids, the cationic charge of the surfactant in the ordered structures is neutralized by the counter ion. Thus, unlike the tail length, the counter ion can have a marked differential impact on the relative stability of surfactant complex with nucleotide with respect to other ordered structures.

Further studies led us to select tetradecyltrimethylammonium oxalate (Catrimox-14) solution as a suitable reagent to isolate RNA from whole blood.

Details of the procedure

The blood sample (100 µl, fresh or anticoagulated with citrate) is mixed with the Catrimox-14 reagent, and transported to the laboratory. The RNA and DNA surfactant complexes are harvested by centrifugation, and the supernatant is carefully removed. The precipitated RNA can be recovered in one of two ways. In the first, the pellet is extracted with hot formamide containing a sodium acetate buffer which preferentially extracts the RNA, leaving most of the DNA as a loose pellet. The RNA is finally precipitated from the formamide by the addition of cold ethanol.

In the second method, the surfactant nucleotide pellet is extracted with 4.0 M guanidinium isothiocyanate, pH 4, which dissociates the complex by virtue of its

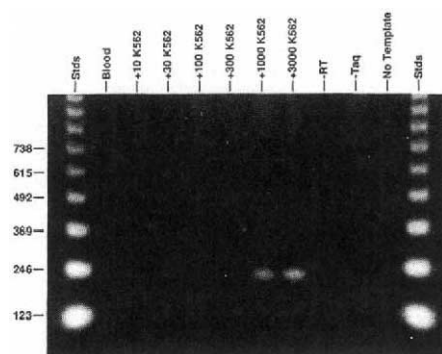


FIG. 2 RT-PCR amplification of *bcr/abl* oncogene. K562 human leukaemia cells (which express the *bcr/abl* hybrid oncogene) were mixed with blood, and (using precautions to prevent contamination¹⁴), the RNA was extracted as described in Fig. 1. The cDNA was made by incubating the RNA at 37 °C for 1 h with 200 units Maloney murine leukaemia virus reverse transcriptase (Gibco BRL) in 40 µl PCR buffer (50 mM KCl; 4 mM MgCl₂; 50 mM Tris, pH 8.4; 100 µg ml⁻¹ bovine serum albumin) containing 20 units RNasin, 5 mM dithiothreitol, 20 pmol downstream primer ('CML-B' of Sawyer *et al.*¹⁵; 5'-TCAGACCCTGAGGCTCAAAGTC-3'), 1mM deoxynucleotide triphosphates (Pharmacia, Piscataway, New Jersey). The reaction was terminated by heating to 95 °C. At this temperature, 80 µl PCR buffer was added, with 20 pmol upstream primer ('C' of Delfau *et al.*¹⁶; 5'-GCTTCTCCTGACATCCGTG-3') and 2.5 units of Taq DNA polymerase (AmpliTaq, Perkin Elmer-Cetus, Emeryville, California). The mixture was overlaid with mineral oil, and amplified by thirty cycles, each consisting of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min in a thermal cycler (Perkin Elmer). The PCR products were examined by ethidium bromide-stained agarose electrophoresis. The predicted amplicon of the *bcr/abl* mRNA cDNA is 219 bp. The lanes are (from left to right): standards (a 123-bp ladder, Gibco BRL); blood alone; blood plus 10, 30, 100, 300, 1,000, 3,000 K562 cells; blood plus 3,000 K562 cells, omitting reverse transcriptase; blood plus 300 K562 cells omitting Taq polymerase; amplification without a template; standards.

ionic strength, and which also inhibits RNase. DNA, residual protein and other impurities are removed from the guanidinium solution by a single phenol-chloroform extraction, and the RNA is recovered by isopropanol precipitation. The formamide method has the advantage of avoiding the use of phenol. The yield of full length RNA is higher with the guanidinium method.

Figure 1 shows an ethidium bromide-stained agarose electrophoresis gel of RNA isolated from whole blood, using the Catrimox-14 surfactant and guanidinium method. The bands of rRNA are clearly seen with the usual smear of other species of RNA. Figure 2 shows the products of RT-PCR using primers that amplify a 219-base pair (bp) segment of the *bcr/abl* gene product in K562 leukaemic cells, which were mixed into normal blood. The expected amplicon was seen by ethidium fluores-

cence when as few as thirty K562 cells were added to the 100 µl blood sample, using only thirty cycles of amplification and a single pair of primers.

Discussion

The use of the cationic surfactant considerably simplifies the extraction of RNA from cells compared with conventional methods. The RNA-surfactant complex is not a substrate for RNase, so that once the blood is mixed with the surfactant, the specimen does not need to be processed immediately. As described above, the cationic surfactant method for whole blood calls for half the number of additions and one or two less centrifugations than the popular 'one-step' method, and it takes less than half the time to perform. RNA isolated from blood by the cationic surfactant method seems to be easy to amplify by RT-PCR, an important consideration because constituents (or their derivatives) of blood tend to inhibit PCR.

The need for a simple method for isolating RNA from whole blood in a form that can be amplified by PCR is great. Appropriate primers may enable the detection and quantitation of RNA encoded by several common pathogens including human immunodeficiency virus, parvovirus,

hepatitis C, cytomegalovirus, mycobacteria, bacteria, and several tropical diseases. Leukocytes express immunoglobulins and several inflammatory mediators (including cytokines, adhesion receptors, and proteins related to blood coagulation) when stimulated, and assaying the corresponding mRNAs¹² may reveal patterns specific for certain autoimmune and other inflammatory diseases. An increasing number of leukaemias and lymphomas are known to have gene rearrangements giving rise to oncogenic mRNAs¹³. Figure 2 shows that a small number of cells with such a rearrangement can easily be detected in a blood sample using the method. Some genetic disorders, such as thalassaemia, may be easier to characterize by analysis of RNA in blood than by other means. Lastly, it is likely to be possible to perform HLA tissue typing and blood group typing by analysis of RNA in blood. □

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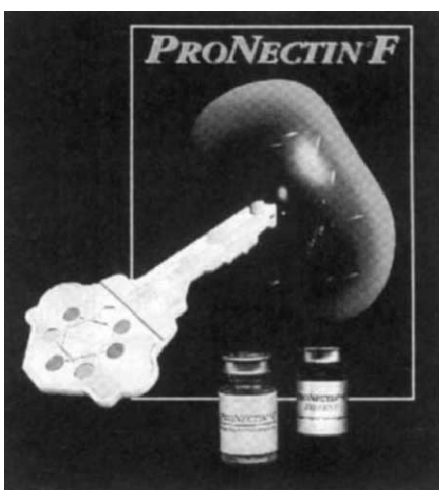
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Trends in tissue culture

An agent to eliminate mycoplasma from cultures, cell attachment factors, media, bioreactors, incubators, filters and a selection of cell and tissue culture catalogues are featured.

ICN Flow announces the availability of MRA, a **mycoplasma removal agent** that ICN developed in conjunction with Dianippon Pharmaceutical of Japan (*Reader Service No. 101*). MRA, a water-soluble oxoquinoline-carboxylic acid derivative, is designed to remove contamination from plant and animal cell cultures and is intended to be useful in research laboratories where valuable cell lines may be at risk. Contaminated cells are cultured with MRA for a week. After removal of the agent, ICN says that the cells will not suffer a recurrent infection with the original mycoplasma. The same agent can also be used as a preventative measure where there may be a danger of mycoplasma contamination in serum or trypsin. The agent is supplied in a 5-ml vial. ICN says that the 50 mg ml⁻¹ solution is sufficient for decontaminating up to 25 cell cultures.

ProNectin F from Protein Polymer Technologies is a genetically engineered **cell attachment factor** that is designed to facilitate the transfer of many anchorage-dependent cell lines from serum-containing



ProNectin F cell attachment factor.

to serum-free media (*Reader Service No. 102*). The company says that use of ProNectin eliminates the need for the laborious 'hit or miss' serum weaning protocols that are usually required to adapt cells to low serum, serum-free or protein-free media.

The PAP pen from Research Products International is a special **marking pen** designed for drawing thin film-like barriers on microscope slides (*Reader Service No. 103*). This barrier creates the proper surface tension needed to hold antibody solution and other specimens within a target area. The pen contains a special water-repellent formulation that is insoluble in alcohol and acetone, and is effective for immunoperoxidase staining, frozen section and fluorescent antibody staining methods. Two models are available: one with a 4-mm tapered point for over 800 applications, and a Mini PAP pen with a 2.5-mm tip for over 400 applications.

A new indicator dye that reacts in response to cellular metabolism and a microplate fluorometer make up the new **cell proliferation assay system** from Alamar (*Reader Service No. 104*). The indicator dye, alamarBlue, produces both a fluorometric and a colorimetric response to cellular proliferation in mammalian cells, bacteria and fungi. It can also be used to establish the relative cytotoxicity of agents



Alamar's cell proliferation/cytotoxicity assay system.

within various chemical classes. The READar microplate fluorometer scans a 96-well microplate in less than 20 s, says Alamar, reading eight wells simultaneously. It is designed to interface with most DOS-based data management systems. Alamar-Blue is designed to replace MTT, XTT, ^3H thymidine and neutral red. The company says that, unlike these reagents, alamarBlue is not toxic to cells, the user or the environment. The company says that it can be incorporated into medium for exposure to cells in excess of 10 days with no adverse effect.

■ Histological helpers

Laboratories that are still using xylene derivatives or paraffin as **clearing agents** may be interested to hear that J. T. Baker has introduced a biodegradable, odourless, non-toxic, noncarcinogenic and chemically inert alternative called Micro-Clear (*Reader Service No. 105*). Micro-Clear is suitable for use with cytochemical and immunoperoxidase techniques. Micro-Cover mountant, a synthetic resin dissolved in toluene, can be used for the coverslipping of histological specimens that have been cleared in Micro-Clear clearing agent.

The Varistain XY is a 21-position, multiprogramme, **robotic batch stainer** available from Shandon (*Reader Service No. 106*). The robotic action of this instrument in two dimensions means that 21 reagents (including water wash) can be accessed in any order. The programme can be up to 50 steps in length and 20, different, schedules can be stored in the memory. The Varistain XY should be useful in laboratories that have to stain batches of slides with various methods, especially special stains. Another application would be in laboratories that would normally have different instruments set up to do various routine tasks.

■ Flasks and substrates

The TripleFlask from Nunc has almost **three times the culture area**, but the same volume, of conventional flasks (*Reader Service No. 107*). The new design offers an expanded culture area of 500 cm² but the same volume as a standard 175 cm² TC NATURE • VOL 362 • 11 MARCH 1993

flask. Filling and emptying of the Triple-Flask is the same as with standard TC flasks: in the upright position the cell suspension levels out; when placed in the incubation position, there will be the same amount of medium in each tray. The TripleFlask is supplied with the same quality certificate as other Nunc TC products, ensuring uniform attachment, spreading and growth of the cells.

Fibra-Cel is Bibby Sterilin's new **cell attachment substrate** for secreted proteins production, which has been developed for use in packed-bed continuous culture, or in stirred batch or roller systems (*Reader Service No. 108*). It consists of 6-mm carrier discs of polyester bound to a polypropylene screen. These can be mixed in a stirred system but as they are designed to be slightly heavier than water they will settle in order to facilitate harvesting. Bibby Sterilin says that Fibra-Cel has been found to facilitate cell growth to densities in excess of 5×10^7 cells per millilitre, which is greater than that achieved by roller bottle or microcarrier techniques. This can be achieved with very low cell-seeding densities, down to one per cent of the final density. When used in a roller bottle or stirred culture system, the Fibra-Cel discs not only increase the surface area for cell attachment but protect fragile cells from shear damage. The material has applications in vaccine and hybridoma production, cellular protein culture and cell biology research.

■ Media mix

TCS Biologicals offers a new Soft Cell range of **serum-free media** (*Reader Service No. 109*). Soft Cell universal is a multipurpose, serum-free medium designed for the cultivation of a wide variety of mammalian cell types. Converting your cells grown on serum to serum-free conditions requires no weaning, according to the company. For hybridoma culture systems, TCS suggests Soft Cell doma, which is available with either a low protein content or as a protein-free formulation. Both are designed to give good levels of hybridoma cell growth and monoclonal antibody production for murine, human and chimaeric hybridomas. For optimum insect cell growth and recombinant protein production there is Soft Cell insecta, which is a ready-to-use protein-free formulation. Soft Cell CHO can be used to optimize the growth of transfected and nontransfected Chinese hamster ovary cells in either monolayer or suspension culture.

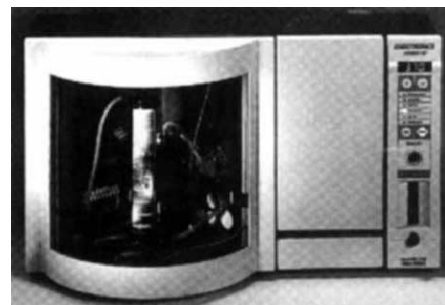
Endothelial cell culture has become an important research tool for the study of the inflammatory response, angiogenesis, wound healing and the action of cardiovascular agents. Human umbilical vein endothelial cell lines are most often used in these applications. Sigma Cell Culture introduces a new line of media and reagents that have been specifically pretested in **endothelial cell cultures** (*Reader Service*

No. 110). Sigma says that these reagents have been refined to work together to create a consistent, dependable cell culture system for human umbilical vein endothelial cell lines and should work with other endothelial-derived cell lines as well. Products in the line include CS-C medium with or without serum, endothelial cell growth factor (100 $\times$), endothelial cell attachment factor, trypsin/EDTA solution for endothelial cell cultures, and trypsin inhibitor for endothelial cell cultures.

Cyberforce is **preselected fetal bovine serum** (FBS) from Sera-Tech which is designed to reduce drastically the FBS concentration in tissue culture systems, rendering serum-dependent cell culture systems (such as transformed cell lines, tumour cells and hybridomas and primary cells) nearly serum-free (*Reader Service No. 111*). No mitogens, hormones, cytokines, tumour-promoting components or vitamins have been added. Because of its heightened biological activity, Cyberforce can be used in a concentration of 0.1 to 1.0 per cent. Sera-Tech recommends a standard concentration of 0.3 per cent. Cells with a need of FBS should be cultured with an additional 0.5 up to 2.0 per cent FBS. Sera-Tech says that, as the FBS content in the culture is reduced considerably when using Cyberforce, the content of undefined components is also reduced. The total protein content of the medium, for example, is reduced to 0.4 mg ml⁻¹ at an application of 0.3 per cent Cyberforce plus one per cent FBS.

■ Bioreactors

Integra Biosciences is distributing a range of **hollow-fibre bioreactors** from Endotronics that are designed to maximize the concentration and relative purity of high-molecular-weight peptides and proteins (*Reader Service No. 112*). The AcuSyst-R system provides researchers with an alternative to roller bottles or *in vivo* systems. It consists of a media reservoir, gas-exchange device, hollow-fibre cartridge and reverse-flow system. The company says that the system can produce 1–3 g of extracellular proteins a month. Dissolved oxygen, temperature, circulation rates and IC/EC cycling are monitored, in addition to six standard input/output pumps, which are both monitored and controlled. Models at the top



Alternative cell culture system for the production of monoclonals.

PRODUCT REVIEW

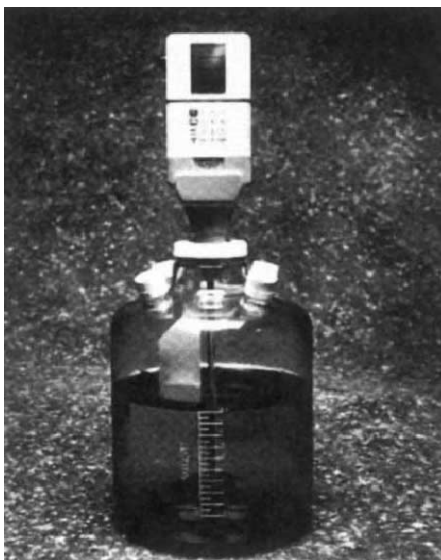
of the range are able to produce 180 g per month.

The CelliGen Plus **bioreactor** from New Brunswick Scientific is capable of producing secreted products and separating cells without the need for internal spin filters or off-line filtration devices (*Reader Service No. 113*). Through the use of a fibrous bed basket, cells remain entrapped in a bed of polyester discs. NBS says that this set-up avoids shear damage typically caused by impellers, gas bubbles and pumps. Cell entrapment allows direct sparging and higher mixing speeds, thus increasing mass transfer of nutrients and oxygen for high product yields, NBS says. The fibrous bed is suitable for the growth of anchorage-dependent cells, without the need for microcarriers, gels or beads. It can also be used with suspension cultures. For cellmass production, a variety of impeller designs may be used to grow most eukaryotic cell lines. The benchtop PC-compatible system is available in 2.2-, 5.0- and 7.5-l capacities.

■ Gadgetry

Heraeus says that precise control over temperature, CO₂ content, humidity and partial oxygen pressure are key elements in the design of its Series 6000 CO₂ **cell culture incubators** (*Reader Service No. 114*). The new air-jacket heating system of the Series 6000 is designed to ensure uniformity of temperature in the internal space and on the walls. Heraeus says that even at a humidity of 97 or 98 per cent, the inner surfaces remain dry. Researchers have a choice of either corrosion-resistant stainless steel or germicidal copper. The copper lining is intended to protect the cultures from bacteria and fungicidal spores, which is a useful feature in multi-user situations where the risk of contamination is often greater. Further protection for sensitive cultures is provided by the encased, plug-in CO₂ sensor, which has a built-in filter. Two models are available with 60- and 220-l capacities.

Nalge's **bioprocess mixing system** is specifically designed for use with the company's 12-l Nalgene culture vessel with



Nalge's bioprocess mixing system.

ports (*Reader Service No. 115*). The system includes an autoclavable culture vessel with ports moulded of lightweight, clear, break-resistant polycarbonate; a 1/30-HP BioTech mixer overhead drive with LED readouts, variable speed controls, programmable timer and audible overload alarm; and a lower assembly featuring a 0.375-inch stainless steel shaft and 4-inch, glass-filled polypropylene axial-flow impeller. The shaft is also fitted with a polypropylene baffle to facilitate top-to-bottom mixing.

Filtropur L is the new biocompatible **pressure filtration unit** with integrated prefilter that is available from Sarstedt (*Reader Service No. 116*). Offering a wide filtration range (from 100 ml to 10 l), these nonpyrogenic, noncytotoxic, high-throughput filters are intended for the filtration of sera and media for cell culture applications. In addition to the fibre prefilter, which, Sarstedt says, is 100 per cent free from binding materials, Filtropur L units are fitted with a wetting agent-free 0.2-µm cellulose acetate membrane that ensures low nonspecific protein binding at high flow rates. A PTFE membrane automatic filtration system allows trapped air to escape,

thus ensuring that the entire filter surface of 20 cm² is utilized.

■ Catalogues

For all your cell culture needs, Intergen announces the availability of a complementary **cell culture products guide** highlighting the company's broad range of reagents (*Reader Service No. 117*). The guide, with its emphasis on applications and references should be a useful tool to any research laboratory using cell cultures. It provides useful information on the company's growth factors and cytokines in tabulated form to assist the researcher. Areas covered include sera, media supplements, products for cell dissociation and recombinant media supplements for growth stimulation.

American Type Culture Collection has printed a 1993 update to the *ATCC Catalogue of Cell Lines and Hybridomas* (7th edn, 1992) (*Reader Service No. 118*). The **cell line supplement** contains descriptions and reference information on the cell lines that became available in ATCC's collection since publication of the 7th edition catalogue in early 1992. The printed update is available free of charge. The complete catalogue and updated information are available on diskette, CD-ROM, and via two online services.

The new 88-page **catalogue** from Harlan Bioproducts for Science features information on the company's more than 2,000 immunological products and services (*Reader Service No. 119*). The company specializes in supplying bulk quantities of sera, growth factors, plasma, organs, glands and tissues. New products include one-bottle chromogens available either in a spray bottle for western blots or as a liquid reagent for ELISAs; a mouse embryo cryopreservation kit; four ELISA kits for testing β-2 microglobulin, IgE or gliadin; and an expanded range of human CD markers. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. For more information, fill in the reader service card bound inside the journal.

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